The Tohoku Journal of Experimental Medicine, Vol. 62, No. 1, 1955

Studies on Tissue Protease

V. Protease Activity of Tissues under Anaphylactic Shock State

(Directed by Asst. Prof. M. Hayakawa)

By

Fumio Ito

(伊藤 文雄)

From Prof. Matunaga's Medical Clinic, Faculty of Medicine, Hirosaki University, Hirosaki

(Received for publication, April 10, 1954)

The activation of serum tryptase system (Plasminogen-plasmin system) under various conditions of stresses has been recognized in relation to pituitary-adrenal system. It is commonly believed that various types of stimuli may cause increased catabolism of body protein. Selye[1] is, however, of the opinion that adrenal cortical hyperactivity is not the causal mechanism of an increased catabolism.

In the preceding paper[2] it was shown that epinephrine caused, independently of adrenal cortex, a rise in protein metabolism in the liver. Westpha[3] showed recently on rat subjected to tourniquet shock or treated with epinephrine that fibrinolytic activity and reduced antifibrinolytc activity could be demonstrated in blood. This may, he considered, be due either to an actual decrease in antiplasmin content or to an augmentation of plasmin content. Nakayama[4] showed that catheptase activity of the kidney was lowered and that ereptase or dipeptidase activity underwent no change under anaphylactic shock state.

Having regard for these findings I undertake certain experiments designed to contribute to knowledge of the problem of protein metabolism in tissue under anaphylactic shock state, measuring protease activities as an indicator.

Experimental

Method and Materials

The method is the same as previously described[2]. Formol titration method is employed using casein, gelatin or pepton as substratum. Increased acidity with respect to each 4 cc. of digestion mixture corrected with values on control experiments (cc. of 0.1 *N* NaOH) appear in the tables.

HEALTH CENTER LIBRARY

Anaphylactic shock: Rabbit sensitized to human blood serum underwent re-injection of 5 cc. antigen through the systemic or the portal vein two or three weeks after the sensitization. Each animal exhibits clinically typical symptom of shock. When immediate death does not occur animals are killed by means of air embolisation.

Results

1. *Proteolysis of liver*

Tryptic hydrolysis of casein by the liver is retarded in the shocked animals, to which antigen is delivered directly through the portal vein, and this is more marked in the case of gelatin hydrolysis. Activation by dialyzed albumin or manganese sulfate or by the both can be observed in such cases and hydrolyses values in activated state are smaller in cases of shocked liver than in the normal ones (Table I, Animals Nos. 50, 51 & 52). Tryptase

TABLE I

Tryptase or Ereptase Activity of the Liver of the Rabbits subjected to Anaphylactic Shock*

Animal No. (kg.)	Time in hrs.	Hydrolysis† at pH 7.8 of						
		Casein (a)	Casein (b)	Casein (c)	Casein (d)	Gelatin (a)	Gelatin (b)	Pepton (a)
40‡ (3.0)	24	0.15	0.3	0.3	0.5	0.1	0.25	0.5
	72	0.2	0.5	0.4	0.8	0.15	0.3	0.65
	120	0.35	0.75	0.7	1.05	0.2	0.55	0.8
41‡ (2.8)	24	0.15	0.4	0.35	0.5	0.1	0.25	0.5
	72	0.2	0.6	0.5	0.85	0.15	0.45	0.65
	125	0.3	0.8	0.65	1.15	0.2	0.55	0.75
42‡ (2.6)	24	0.1	0.3	0.3	0.55	0.05	0.2	0.55
	72	0.25	0.65	0.45	0.9	0.1	0.4	0.6
	124	0.3	0.85	0.7	1.15	0.2	0.45	0.65
50§ (3.2)	24	0.05	0.2	0.2	0.2	0.05	0.2	0.35
	72	0.1	0.3	0.25	0.5	0.05	0.3	0.55
	120	0.15	0.45	0.4	0.7	0.1	0.35	0.6
51§ (2.8)	24	0.1	0.15	0.2	0.2	0.05	0.15	0.4
	72	0.2	0.35	0.3	0.45	0.05	0.15	0.65
	120	0.2	0.4	0.4	0.6	0.1	0.3	0.65
52§ (3.0)	24	0.05	0.2	0.2	0.25	0.05	—	0.4
	72	0.15	0.3	0.25	0.45	0.1	—	0.55
	120	0.2	0.4	0.4	0.65	0.1	—	0.6
Normal//	24	0.15	0.3	0.35	0.5	0.1	0.25	0.7
	72	0.25	0.55	0.5	0.9	0.2	0.4	1.0
	120	0.35	0.7	0.7	1.1	0.3	0.65	1.1

* Reinjection: 5 cc. human serum.

† (a) Without albumin or manganese sulfate solution; (b) With dialyzed ovalbumin solution; (c) With manganese sulfate solution; (d) With dialyzed ovalbumin and manganese sulfate solutions.

‡ Re-injection of antigen is through the systemic vein.

§ Re-injection of antigen is through the portal vein.

// Average of five animals (Nos. 35, 36, 45, 46 and 47).

TABLE II

Catheptase Activity of the Liver of the Rabbits subjected to Anaphylactic Shock*

Animal No. (body weight, kg.)	Time in hrs.	Hydrolysis† at pH 4.5 of					
		Casein		Gelatin		Pepton	
		(a)	(b)	(a)	(b)	(a)	(b)
40‡ (3.0)	24	0.15	0.5	0.15	0.4	0.2	0.5
	72	0.3	0.65	0.25	0.6	0.4	0.6
	120	0.4	0.65	0.35	0.65	0.6	0.75
41‡ (2.8)	24	0.15	0.5	0.2	0.35	0.3	0.45
	72	0.35	0.55	0.3	0.6	0.4	0.6
	120	0.4	0.6	0.4	0.65	0.6	0.75
42‡ (2.6)	24	0.15	0.35	0.2	0.4	0.35	0.45
	72	0.3	0.6	0.3	0.6	0.4	0.4
	120	0.4	0.65	0.4	0.65	0.6	0.7
50‡ (3.2)	24	0.1	0.2	0.15	0.3	0.15	0.25
	72	0.2	0.4	0.2	0.45	0.3	0.55
	120	0.3	0.5	0.35	0.6	0.35	0.55
51‡ (2.8)	24	0.1	0.25	0.15	0.35	0.2	0.35
	72	0.25	0.5	0.25	0.45	0.3	0.5
	120	0.35	0.55	0.35	0.6	0.4	0.5
52‡ (3.0)	24	0.1	0.3	—	—	0.2	0.35
	72	0.25	0.4	—	—	0.3	0.6
	120	0.35	0.55	—	—	0.35	0.65
Normal§	24	0.25	0.45	0.2	0.5	0.35	0.5
	72	0.35	0.6	0.35	0.6	0.55	0.75
	124	0.45	0.75	0.4	0.7	0.65	0.8

* Re-injection: 5 cc. human serum.

† (a) Without cysteine; (b) With cysteine activation.

‡ Re-injection of antigen is through the systemic vein.

§ Re-injection is through the portal vein.

// Average of three animals (Nos. 45, 46 and 47).

TABLE III

Autolytic Proteolysis of the Liver of the Rabbits subjected to Anaphylactic Shock

pH of autolysis	Time in hrs.	Autolytic Proteolysis of the Liver of Animal*						
		No. 40	No. 41	No. 42	No. 50	No. 51	No. 52	Normal+
7.8	24	0.3	0.3	0.25	0.2	0.25	0.25	0.35
	72	0.4	0.45	0.45	0.3	0.35	0.35	0.55
	120	0.75	0.8	0.75	0.6	0.6	0.65	0.85
4.5	24	1.3	1.25	1.2	1.2	1.2	0.25	1.5
	72	2.0	1.95	2.05	1.8	1.9	1.85	2.15
	120	2.3	2.25	2.25	2.0	2.05	2.05	2.5

* Cf. Table I, Footnotes.

† Average of four animals (Nos. 45, 46, 47 and 64).

TABLE IV

Tryptase or Ereptase Activity of Rabbit Liver delivered with Acetylcholine or kept Anoxic

Animal No. (body weight, kg.)	Time in hrs.	Hydrolysis* at pH 7.8 of						
		Casein				Gelatin		Pepton
		(a)	(b)	(c)	(d)	(a)	(b)	(a)
54† (2.6)	24	0.2	0.35	0.4	0.55	0.1	0.2	0.95
	72	0.3	0.5	0.4	1.0	0.1	0.25	1.1
	120	0.4	0.7	0.95	1.05	0.2	0.35	1.2
56† (3.2)	24	0.15	0.3	0.35	0.4	0.15	0.15	0.7
	72	0.3	0.5	0.45	0.9	0.2	0.45	1.0
	120	0.4	0.7	0.9	1.1	0.2	0.45	1.25
60‡ (3.7)	24	0.25	0.5	0.3	0.75	0.2	0.4	0.75
	72	0.55	0.8	0.8	1.2	0.3	0.5	1.1
	120	0.65	1.1	1.05	1.4	0.35	0.6	1.25
65‡ (2.0)	24	0.25	0.5	0.35	0.7	0.1	0.25	0.75
	72	0.35	0.75	0.7	1.25	0.25	0.45	1.1
	120	0.5	1.05	1.1	1.45	0.25	0.6	1.2
66‡ (1.9)	24	0.3	0.45	0.5	0.75	0.15	0.2	0.8
	72	0.5	0.8	0.75	1.2	0.25	0.5	1.15
	120	0.65	1.1	1.05	1.55	0.35	0.6	1.15
55§ (3.0)	24	0.15	0.3	0.35	0.5	0.05	0.15	0.8
	72	0.35	0.55	0.45	0.85	0.1	0.3	1.05
	120	0.35	0.6	0.75	1.15	0.15	0.35	1.05
Normal//	24	0.15	0.3	0.35	0.5	0.1	0.25	0.7
	72	0.25	0.55	0.5	0.9	0.2	0.4	1.0
	120	0.35	0.7	0.7	0.1	0.3	0.65	1.1

* Without albumin or manganese activation; (b) With dialyzed serum albumin solution; (c) With manganese activation; (d) With combined activation with dialyzed serum albumin and manganese sulfate solutions.

† Delivery is through the portal vein (No. 54, 25 mg.; No. 56, 100 mg.).

‡ Delivery is through the systemic vein (No. 60, 50 mg.; No. 65, 5 mg.; No. 66, 10 mg.).

§ Died 12 hours after the ligation of all blood vessels of liver.

// Cf. Table I, Footnotes.

activity in shocked liver is, therefore, lowered as a whole, and in such cases ereptase activity (peopn, pH 7.8) is lowered too (Table I, Animals Nos. 50, 51 & 52). Catheptic hydrolysis of casein or pepton is also retarded in shocked liver, and this is also the case when comparison is made under cysteine activation, but the retardation of gelatin hydrolysis is less marked (Table II, Animals Nos. 50, 51 & 52). At any rate catheptase activity of shocked liver is lowered too. Autolysis at pH 4.5 is also apparently retarded in the shocked livers and that at pH 7.8 too (Table III, Animals Nos. 50, 51 & 52). However, when antigen is reinjected through the systemic vein, retardation of protease activity can scarcely be observed

(Tables I, II, & III, Animals Nos. 40, 41 & 42).

Next I examined the effect of acetylcholine, because it is suspected by some scholars to be a causal factor in the anaphylactic shock. When it is delivered through the portal vein, activity of protease system undergoes no change although the animals immediately die from shock (Tables IV, V & VI, Animals Nos. 54 & 56). In such cases no differences in protease

TABLE V

Catheptase Activity of Rabbit Liver delivered with Acetylcholine or kept Anoxic

Animal No. (body weight, kg.)	Time in hrs.	Hydrolysis* at pH 4.5 of					
		Casein		Gelatin		Pepton	
		(a)	(b)	(a)	(b)	(a)	(b)
54† (2.6)	24	0.25	0.45	0.3	0.45	0.4	0.55
	72	0.45	0.75	0.35	0.65	0.5	0.8
	120	0.6	0.85	0.4	0.65	0.6	0.85
56† (3.2)	24	0.25	0.5	0.25	0.45	0.35	0.6
	72	0.45	0.65	0.3	0.6	0.55	0.7
	120	0.55	0.85	0.35	0.7	0.55	0.8
60‡ (3.7)	24	0.6	0.75	0.65	0.9	0.55	0.85
	72	1.05	1.55	0.75	1.35	0.7	1.2
	120	1.2	1.85	0.9	1.35	0.9	1.3
65‡ (2.0)	24	0.35	0.65	0.3	0.6	0.4	0.7
	72	0.75	1.15	0.45	0.85	0.7	0.85
	120	0.8	1.3	0.6	1.0	0.8	1.05
66‡ (1.9)	24	0.35	0.6	0.45	0.65	0.5	0.65
	72	0.75	1.1	0.45	0.9	0.7	0.8
	120	0.85	1.25	0.6	0.95	0.9	1.05
55§ (3.0)	24	0.25	0.4	0.25	0.4	0.35	0.55
	72	0.35	0.55	0.35	0.55	0.45	0.65
	120	0.4	0.75	0.4	0.65	0.45	0.75
Normal//	24	0.25	0.45	0.2	0.5	0.35	0.5
	72	0.35	0.6	0.35	0.6	0.55	0.75
	120	0.45	0.75	0.4	0.7	0.65	0.8

* (a) Without cystein; (b) With cystein activation.

† Delivery is through the portal vein. (No. 54, 25 mg.; No. 56, 100 mg.).

‡ Delivery is through the systemic vein. (No. 60, 50 mg.; No. 65, 5 mg.; No. 66, 10 mg.).

§ Died 12 hours after ligation of all blood vessels of liver.

// Cf. Table II, Footnotes.

activities between the left and right lobulus of the liver was observed. But when delivery is through the systemic vein animals die in similar manner and amount of heterolytic or autolytic hydrolysis becomes higher and augmentation of tryptase or catheptase is concluded (Tables IV, V & VI, Animals Nos. 60, 65 & 66).

The protease activities of the liver which beforehand is kept anoxic after ligation of all blood vessels are proved to be unchanged (Tables IV, V & VI, Animal No. 55). This animal died 12 hours after ligation.

Histological examinations reveal blood congestion, cell degeneration

TABLE VI

Autolytic Proteolysis of Rabbit Liver delivered with Acetylcholine or kept Anoxic

pH of autolysis	Time in hrs.	Autolytic Proteolysis of the Liver of Animal*						
		No. 54	No. 56	No. 60	No. 65	No. 66	No. 55	Normal†
7.8	24	0.55	0.55	0.55	0.6	0.5	0.35	0.35
	72	0.75	0.7	0.8	0.8	0.85	0.5	0.55
	120	1.05	1.05	1.1	1.05	1.05	1.05	0.85
4.5	24	1.7	1.7	1.75	1.75	1.7	1.4	1.5
	72	2.35	2.3	2.35	2.3	2.25	2.1	2.15
	120	2.75	2.65	2.8	2.85	2.8	2.4	2.5

* Cf. Table I, Footnotes.

† Average of four animals (Nos. 45, 46, 47 and 64).

TABLE VII

Proteolysis of the Kidney or the Gastric mucosa of the Rabbits subjected to Anaphylactic Shock or delivered with Acetylcholine

A. Proteolysis of the kidney

Animal*	Time in hrs.	Hydrolysis at pH 7.8				
		without activation			with albumin activation†	
		Casein	Gelatin	Pepton	Casein	Gelatin
(A)	24	0.3	0.25	0.65	0.65	0.6
	72	0.85	0.55	0.9	1.8	1.05
	120	1.0	0.9	1.05	2.15	1.7
(B)	24	0.25	0.1	0.55	0.45	0.2
	72	0.65	0.3	0.75	1.2	0.55
	120	0.8	0.4	0.75	1.6	0.85
(C)	24	0.45	0.3	1.0	0.85	0.65
	72	1.0	0.7	1.3	2.45	1.6
	120	1.3	1.1	1.25	2.75	2.1
(D)	24	0.35	0.35	1.1	0.8	0.75
	72	0.8	0.7	1.2	1.7	1.75
	120	1.2	1.15	1.3	2.6	2.25
(E)	24	0.4	0.35	1.15	0.8	0.65
	72	1.0	0.7	1.25	2.3	1.95
	120	1.1	1.1	1.25	2.7	2.45
Normal‡	24	0.35	0.35	1.1	0.65	0.75
	72	1.05	0.7	1.25	2.1	1.65
	120	1.25	1.1	1.25	2.65	2.25

B. Proteolysis of the gastric mucosa

Animal* No.	Time in hrs.	Hydrolysis at pH 7.8				
		without activation			with albumin activation†	
		Casein	Gelatin	Pepton	Casein	Gelatin
(A)	24	0.15	0.1	0.4	0.3	0.25
	72	0.35	0.4	0.6	0.75	0.8
	120	0.4	0.45	0.65	0.9	1.0
(B)	24	0.15	0.1	0.35	0.35	0.25
	72	0.4	0.35	0.55	0.8	0.75
	120	0.45	0.5	0.55	1.0	1.05
(C)	24	0.15	0.1	0.45	0.3	0.25
	72	0.4	0.35	0.55	0.9	0.75
	120	0.45	0.5	0.6	1.0	1.1
(D)	24	0.25	0.1	0.4	0.55	0.3
	72	0.35	0.4	0.5	0.75	0.75
	120	0.45	0.5	0.55	0.95	1.1
(E)	24	0.2	0.1	0.35	0.45	0.25
	72	0.35	0.4	0.5	0.75	0.8
	120	0.4	0.5	0.6	1.0	1.2
Normal‡	24	0.15	0.1	0.35	0.35	0.25
	72	0.35	0.4	0.55	0.85	0.8
	120	0.4	0.5	0.55	1.0	1.0

* (A), Antigen is reinjected through the systemic vein : Average of three animals Nos. 40, 41 & 42. (B), Antigen is reinjected through the portal vein : Average of three animals Nos. 50, 51, & 52. (C), Acetylcholine delivery through the portal vein : Average of two animals Nos. 54 & 56. (D) Acetylcholin delivery through the systemic vein : Average of three animals Nos. 60, 65 & 66. (E), Ligation of all blood vessels of the liver, animal No. 55.

† Dialyzed serum albumin solution is used.

‡ Average of three animals Nos. 45, 46 & 47.

in shocked liver, and blood congesiton, small hemorrhage or degeneration in liver delivered with acetylcholine, and distension of sinusoids, hemorrhage and degeneration of liver cells in anoxic liver.

2. *Proteolysis of the gastric mucosa or of the kidney* (Table VII)

Neither retardation nor activation of the proteolytic activity of the gastric mucosa is observed under anaphylactic shock state or by acetylcholine delivery or by anoxic state of the liver. But it is curious that the activities of the tryptase and ereptase of the kidney of the shocked animals which received antigen through the portal vein is, as well as those of the liver, apparently lowered. In the case of the acetylcholine delivery, either through the systemic or the portal vein, no activation of the kidney protease is observed. Ligation of the blood vessels of the liver exerts no influence upon proteolysis of the kidney.

Discussion

Under various states of stresses catabolism of body protein is believed to take place, and corresponding to this the activation of the protease system in any tissue might, under such condition, be anticipated. Contrary to the above anticipation, however, retardation of the activity of the liver protease system is observed on the animals reinjected with antigen through the portal vein and subjected to anaphylactic shock state. In such cases, unexpectedly, retardation of kidney tryptase and ereptase activities is observed simultaneously. This may suggest some physiological correlation between liver and kidney. Such retardation can not be simply attributed to the result of blood congestion which renders liver anoxic, because the latter condition exerted no change in protease activities of the liver. When activity of protease system in tissue is taken as a direct indicator for progressive protein metabolism, it may be concluded that protein metabolism, either catabolism or anabolism, in the liver or in the kidney is, in such occasion, simultaneously lowered. Therefore liver or kidney is not likely to play a main part for catabolism of body protein under anaphylactic shock state. Because of relatively manifest degeneration of liver parenchymatous cells it may be also concluded that cellular damage caused by shock results in such retardation of enzymatic activities. On the other hand, the activation of the protease system of the liver after delivery of acetylcholine might have resulted from the releasing of epinephrine, because acetylcholine is well known for activating the adrenal system and the latter is proved to be activating for the liver protease system[2]. Such animals, receiving acetylcholine immediately dies from shock state, and histological examination of the liver reveals the same findings as those of anaphylactic livers. Nevertheless it is of interest that the behavior of the liver protease system in anaphylactic shock differs markedly from that in acetylcholine shock.

Summary

1. Tryptase, ereptase, catheptic proteinase and peptonase activities are lowered in the liver under anaphylactic shock state, when antigen is reinjected through the portal vein directly to the liver. In such cases tryptase and ereptase activities in the kidney are lowered too. But when antigen is reinjected through the systemic vein, such retardation of the protease activities is not so manifest.

2. Delivery of acetylcholine through the systemic vein produces activation of the liver protease system but through the portal vein it produces no change.

3. Anoxic state of the liver produces neither retardation nor activation of the protease system in the liver or in the kidney.

4. Gastric mucosa proteolysis undergoes no change under each of above conditions.

5. Liver or kidney is not likely to play the main role in protein catabolic response to stimuli such as anaphylactic shock state.

This work was carried out by a grant for development of scientific researches, given from the Ministry of Education. M. Hayakawa

References

1) Selye, Textbook of Endocrinology, 2nd Ed., Montreal, 1949.
2) Ito, Tohoku J. Exp. Med., 1955, **61**, 399.
3) Westphal *et al.*, Proc. Soc. Exp. Biol. & Med., 1950, **75**, 862.
4) Nakayama, Tohoku J. Exp. Med., 1940, **38**, 537.

The Tohoku Journal of Experimental Medicine, Vol. 62, No. 1, 1955

Studies of High-molecular Placental Components in Connection with Pregnancy Toxemia

Fourth Paper

On Placental Non-Hexuronic Acid-Containing Mucopolysaccharide*

By

Yosizi Mugikura
(麥倉 義司)

From the Medico-chemical Institute (Director: Prof. H. Masamune) and the Department of Obstetrics and Gynecology (Director: Prof. T. Shinoda), Tohoku University, Sendai

(Received for publication, April 15, 1954)

In continuation of the former work[1], the mucopolysaccharide of placenta containing no hexuronic acid was prepared according to the new scheme to apply the placental high-molecular components in a state as native as possible to the biological assays. The substance obtained, which was essentially homogeneous electrophoretically and analyzed as will be described, proved less effective even than the placental DNA or non-effective at all in causing pregnancy toxemia.

Experimental

Preparation Procedure of the Mucopolysaccharide

Fresh placentas, washed with water and freed from membranes, were cut into small pieces, squeezed and ground in a masticator. The brei was extracted with 1.5, 1.4 and 1.2 volumes of 0.14 *M* NaCl containing 0.01 *M* sodium citrate at 0°C, extending over 24 hours each time. The red-colored, turbid mixture (pH 6.6–6.8) of the solutions was acidified with dilute HCl to pH 4.2, whereby a bulky precipitate occurred, and after standing for several hours, centrifuged. The centrifugate was set aside to separate itinsulfuric acid, pentosenucleic acid and a protein. The transparent, dark brown supernatant was neutralized with a dilute NaOH solution, whereby again a precipitate occurred. The centrifuged supernatant here, that was transparent and red-colored, was distilled in vacuo to 1/10—1/15 the volume, saturated with $(NH_4)_2SO_4$, and adjusted to pH 4.2 with dilute H_2SO_4, followed by standing overnight. Filtered. The

* The 177th communication of Masamune and coworkers' " Biochemical Studies on Carbohydrates ".

mass on the filter was reprecipitated three times similar to above, dissolving in water by means of a homogenizer. The four successive filtrates were combined, dialyzed against running tap water for 3–4 days and distilled under diminished pressure to dryness, rejecting halfway the precipitates during dialysis and concentration. The still-residue showed strong Molisch-, moderate biuret- and weak Neuberg-Saneyoshi-reaction. Phosphorus was detected. After several runs with 15,357 g. in total of the starting batches of brei (47 placentas), 4.5 g. of this fraction were given.

The brown products united were kneaded with the sat. $(NH_4)_2SO_4$, filled up to 150 cc. with the same salt solution and acidified with 10% H_2SO_4 to pH 4.2 as above. After 6 hours the insoluble part was centrifuged and subjected to similar treatment with the sat. $(NH_4)_2SO_4$ three more times. The solutions thus obtained were combined and dialyzed for 1 week, then the precipitate that appeared during dialysis was removed and the fluid was concentrated by vacuum distillation to about 150 cc. The somewhat cloudy condensate (pH 6.0–6.2) was added to with 10% lead acetate (crystalline) in portions till no more occurrence of precipitate, and after standing overnight, filtered. The filtrate was dialyzed for 3 days and condensed to about 100 cc. and as much 20% barium acetate (crystalline) was added as to carry down all the precipitable, followed by alkalization of the mixture to pH 8.8 with a few drops of the sat. $Ba(OH)_2$. The centrifuged supernatant here was precipitated by addition of alcohol to 50% (The precipitate freed from barium gave positive Molisch, Goldschmiedt, Neuberg-Saneyoshi and biuret reactions) and centrifuged, and the supernatant was distilled in vacuo to about 1/3 the volume to expel off the alcohol. The fluid was further freed from barium by the aid of 10% H_2SO_4 (This was added to pH 1.0), dialyzed for 1–2 days and after filtration, passed through columns of Amberites IRA-410 and IR-120 alternatively. When its change in pH ceased, it was condensed by vacuum distillation to a syrup, which was precipitated with an alcohol-ether (5 : 1 by volume) mixture and dried in vacuo over $CaCl_2$. Yield 323 mg. Molisch ##, biuret ±, phosphorus −, Goldschmiedt +, Neuberg-Saneyoshi −.

187 mg. of the substance given were taken up in 16 cc. of water, and added to with 2 cc. of 20% barium acetate (crystalline) and subsequently with abs. alcohol to 60%. Some precipitate, which was ropy and adhered to the tube on centrifugation, was discarded, because it was biuret + and Goldschmiedt ±, although it was Molisch ##. The supernatant was freed from most of alcohol, passed through the Amberites, condensed, precipitated and dried as above. 100 mg. of a white powder were given.

Physical and Chemical Properties

Homogeneity. It proved essentially homogeneous electrophoretically.

The patterns of it when examined at pH values of 7.7 and 4.5 are shown in Fig. 1.

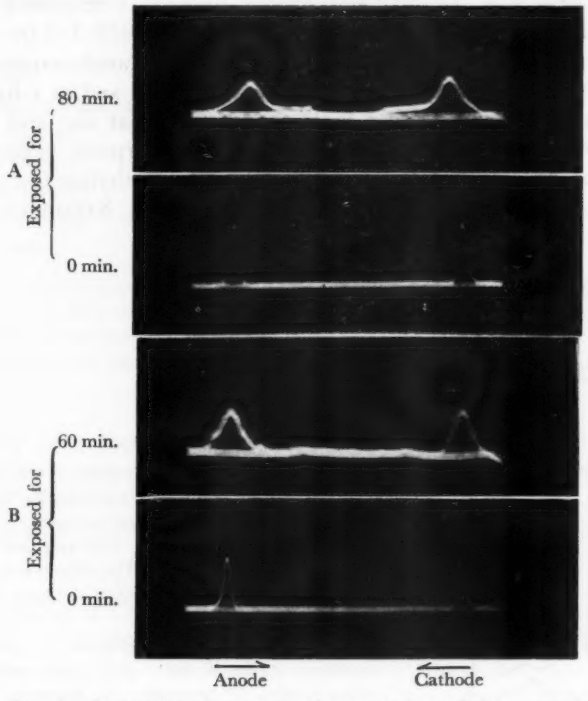

Fig. 1. Electrophoretic patterns of 1% solutions of the mucopolysaccharide in phosphate buffer pH 7.7, I 0.2 (A) and acetate buffer pH 4.5, I 0.2 (B); temperature 10°C; current 8 mA; mobility in the phosphate buffer $=\frac{0.21\times0.0065\times0.3}{4800\times0.008}=1.07$ cm^2•volt^{-1}•sec^{-1}•10^5 (calculated as to the descending boundary); mobility in the acetate buffer = 0. The ascending limb in A is to the left.

Test tube tests. Molisch ## ; Bial-Mejbaum ##, indirect Osaki-Turumi +, direct Osaki-Turumi ##, hydrolysable sulfur detected; Neuberg-Saneyoshi, Goldschmiedt and biuret negative; phosphorus and glycogen (iodine test) were absent.

Precipitability. Neither of cupric chloride, lead acetate, barium acetate and ammonium sulfate precipitated it from water.

Paper partition chromatography. 3 mg. of the substance were hydrolyzed with 0.3 cc. of 1 *N* HCl in an ampoule (Temperature and duration of hydrolysis 100°C and 5 hrs. respectively). The centrifuged supernatant

was freed from HCl as far as possible according to Masamune *et al.*[2] The dried residue was taken up in 0.1 cc. of water and neutralized, and 0.05 cc. of the solution was sampled and further treated as described by those authors (Irrigation solvent: butanol-pyridine-water (5:3:2 by volume)). Spots of a probable disaccharide or disaccharides, chondrosamine (weak), glucosamine, galactose, glucose, mannose and xylose and/or L-fucose were given (Fig. 2). Xylose and L-fucose are localized at one and the same position by the solvent used. However, no absorption suggesting the presence of pentose but that corresponding to methylpentose occurred, when the substance was subjected to Masamune & Sakamoto's spectro-

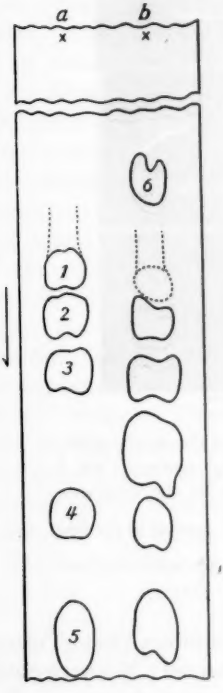

Fig. 2. Sugar paper-chromatogram of the mucopolysaccharide diagrammatically reproduced. Descending irrigation with butanol-pyridine-water (5:3:2 by volume) at 23°C for 22 hrs. Indicator aniline hydrogen phthalate of Partridge. The reference sugar solution was 1% with respect to individual sugars and 0.02 cc. of it was sampled.

a: reference run, b: main run. 1: chondrosamine hydrochloride, 2: glucosamine hydrochloride, 3: galactose, 4: mannose, 5: L-fucose, 6: probable unit disaccharide or disaccharides.

photometric method of analyzing sugars.[3] Therefore the spot in question must have been the one of L-fucose.

Quantitative analyses. See Table I.

Optical rotation in water. $[\alpha]_D^{\circ} = \frac{+0.964 \times 1}{0.0101 \times 1} = +95.4^{\circ}$

TABLE I

Composition of the Mucopolysaccharide

Analysis	In per cent	In equivalents per equivalent weight
N*	2.5	2.9
Hexosamines as glucosamine†	11.0	1.0
Galactose‡	8.2	0.7
Glucose‡	74.6¶	6.7
Mannose‡	6.7	0.6
L-Fucose§	1.9	0.2
Ash//	3.0	

* Micro-Kjeldahl, † Blix, ‡ Masamune & Sakamoto[3], § Masamune & Sakamoto[4], // Pregl without use of H_2SO_4, ¶ The iodine test disproved the presence of glycogen in the substance as described in the text, despite of the high glucose content.

Biological Assays

By the procedures of Kanno *et al.*[5] were given the results summarized below. The details will be published by Drs. Funakubo[6] and Saito[7].

1) Pathological changes in liver and kidney of pregnant rabbits and symptoms after injection of the substance are embodied in Table II.

TABLE II

Pathological Changes in Liver and Kidney of Pregnant Rabbits after Injection of the Mucopolysacchride

5 mg. per kg. body weight of the substance were injected into auricular vein as a 1% solution in physiological saline. $-\sim++$ with respect to histological changes signify the same grades of changes as the corresponding notations in the paper of Kanno *et al.*[5]

Rabbit No.	Rabbit Wt. (g.)	Days of pregnancy	Time of killing animal after inj. (hrs.)	Liver: Necrosis	Liver: Necrobiosis	Liver: Cloudy swelling	Liver: Degeneration	Liver: Stasis of blood str.	Liver: Thrombosis	Liver: Change of Glisson capsule†	Kidney: Nephrosis	Kidney: Glomerulonephrosis	Premature delivery‡	Aggrav.§ of general status aft. inj.
70	3150	18	41	−	−	±	+	−	−	+	−	−	+	± + +
71	3100	28	48	−	−	−	++	−	−	+	−	−	−	
72	2800	25	48	−	−	−	+	−	−	−	−	−	−	
Contr. 1*	2900		49	−	−	−	−	−	−	+	−	−		
Contr. 2*	2360		45	−	−	+	−	±	−	+	−	−		− ± + ±

* A non-pregnant female rabbit was injected with the mucopolysaccharide solution. † Cellular infiltration and swelling of the sheath. ‡ + signifies the occurrence, § − : no aggravation, ± : faint aggr., + : slight aggr.

There was found no distinct discrepancy of histological changes between the pregnant and non-pregnant female rabbits, although degeneration of liver cells in pregnant rabbits appears to indicate some tendency of intoxication together with slight aggravation of general status and premature delivery.

2) Blood pressure of pregnant rabbits is not influenced as was revealed in the findings in Table III.

TABLE III

Blood Pressure in Carotic Artery of Pregnant Rabbits after Injection of the Mucopolysaccharide

5 mg. per kg. body weight of the substance were injected as a 1% solution (in physiological saline) into auricular vein of urethane-narcotized pregnant rabbits.

Rabbit No.	Rabbit Wt. (g.)	Days of pregnancy	Blood pressure in mm. Hg					
			Before injection	Time after injection in min.				
				1/2	1	3	5	7
73	3150	18	95	96	97	96	98	95
74	3100	28	110	112	110	113	112	114
Control*	2900		108	110	110	109	108	111

* A non-pregnant female rabbit was injected with the mucopolysaccharide solution.

3) No change of urine volume nor albuminuria happened, when a pregnant rabbit was intravenously (into auricular vein) injected with 5 mg. per kg. body weight of the substance, simultaneously administering 100 cc. of water into stomach.

4) Precipitin reaction. The test was made with sera of 5 each of pregnancy-toxemic, normal pregnant and non-pregnant women. In each group of the sera, 1 gave positive reaction at a dilution of 1:100 of the substance as the antigen but the others not.

To summarize, the present mucopolysaccharide plays, even if it can, only a minute part of causing pregnancy toxemia.

Through the Grant Committee for Scientific Researches the Education Department gave a grant in aid to us, which is gratefully acknowledged.

H. Masamune

References

1) Mugikura, Tohoku J. Exp. Med., this volume, p. 261.
2) Masamune, Hakomori, Numabe, Akama & Kamiyama, *ibid.*, this volume, p. 283.
3) Masamune & Sakamoto, *ibid.*, in press.

4) Masamune & Sakamoto, Tohoku J. Exp. Med., in press.
5) Kanno, Kimura, Tanaka & Mugikura, *ibid.*, 1954, **60**, 257.
6) Funakubo, Nippon Sanka-Fujinka Gakkai Zasshi (Jap.), in press.
7) Saito, *ibid.*, in press.

Note at proof-reading: Funakubo's and Saito's articles (6) and 7)) have been published in that Journal (1955, **7**, 89 and 691).

The Tohoku Journal of Experimental Medicine, Vol. 62, No. 1, 1955

Biochemical Studies on Carbohydrates

CLXXVIII. On Molecular Aggregation in Solution of a Group-active Mucopolysaccharide from Pig Stomach Mucus

By

Yuh-iti Suzuki
(鈴木 佑一)

From the Medico-chemical Institute, Tohoku University, Sendai;
Director: Prof. H. Masamune

(Received for publication, April 15, 1954)

The group mucopolysaccharide, which is obtainable by scission with 1 *N* NaOH at 20°C of pig stomach mucus, has been studied exhaustively regarding its chemical structure by Masamune and Yosizawa[1]. And furthermore its spacial configuration was established by Masamune *et al.*[2] It appears thus to be a suitable material for investigating the aggregate state of mucopolysaccharides in solution. The substance in phosphate buffer of pH 7.0 and I 0.147 was found to have the average axial ratio of 19/1 as anhydrous rods or 51/1 as anhydrous disks from viscosity measurement, and molecular weight M of 160,000, as calculated from osmotic pressure, or either 170,000 as rods or 90,000 as disks, as calculated from diffusion constant and axial ratio.

According to the authors above, the molecular weight chemically estimated *m* of this mucopolysaccharide is approximately 3,600[2] and the carbohydrate moiety forms a coil, of which one of the cut ends is shielded by a peptide chain. If the molecule is closely packed, as is most possible, its model forms a cylinder of axial ratio not far diverging from 1/1. A disk of M of 90,000 is made by aggregation of about 25 of the units but can not have an axial ratio of 51/1 by all means, so that the unit molecules are regarded as associated into rods in the solution. An aggregate of M of 160,000–170,000 is made with 46 in averages (44–47) of the units. If a half of the rods are single threads made probably by head-to-tail arrangement of 19 units in averages and the others are two threads attached together laterally, in each of which 38 units in averages are associated longitudinally as above, the mean values of M and axial ratio of the rods come to 170,000 and 19:1 respectively.

Those rods are presumably fragmentized by the agglutinin to combine with the latter, when a solution of the group mucopolysaccharide is mixed with the antiserum, because it is conceived that the agglutinin possesses

stronger affinity to the bare cut end of the carbohydrate coil in a unit molecule than the peptide chain in another unit.

Experimental

Material Employed

The mucopolysaccharide was prepared as Yosizawa described[3], and dried to a constant weight in vacuo over $CaCl_2$. It proved electrophoretically essentially homogeneous as shown in Fig. 1. Biuret faintly positive,

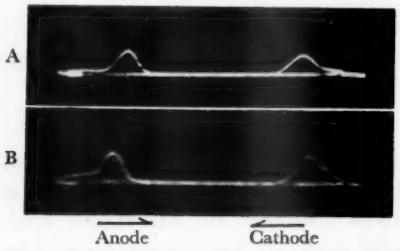

Fig. 1. Electrophoresis patterns of 1% solutions of the mucopolysaccharide in phosphate buffer pH 7.8, I 0.144 (A) and acetate buffer pH 4.7, I 0.10 (B); temperature 10° and 12°C respectively; current 10 mA; exposure after starting current 35 and 30 minutes respectively. Ascending limbs to the left.

Molisch strongly positive, indirect Osaki-Turumi positive, direct Osaki-Turumi almost negative, P not detected. Analysis: N (micro Kjeldahl) 4.51%, glucosamine (Blix)[4] 38.0%, galactose (Masamune & Sakamoto[5]) 29.8%, L-fucose (Masamune & Sakamoto[6]) 8.6%, ash (Pregl without use of H_2SO_4) 0.51%. It was Group A and Group O active at dilutions of $1:10^6$ and $1:10^5$ respectively. The serological assay was processed according to Masamune *et al.*[7] by employing a glass plate for the hemagglutination. Group O activity was measured with an eel serum.

Physical Examination

The following experiments were carried out with solutions in phosphate buffer pH 7.0, I 0.147.

Axial ratio calculated from viscosity

Simha[8] postulated that equations below hold for anhydrous prolate and oblate ellipsoids (rods and disks):

$$\text{Rods:}\quad \nu=\frac{f^2}{15\,(\ln 2f-\frac{3}{2})}+\frac{f^2}{5\,(\ln 2f-\frac{1}{2})}+\frac{14}{15} \qquad \text{(1a)}$$

$$\text{Disks:}\quad \nu=\frac{16}{15}\,\frac{f}{\tan^{-1}f} \qquad \text{(1b)}$$

where f is axial ratio a_1/a_2 in (1a) and a_2/a_1 in (1b), and ν volume intrinsic viscosity. On the other hand, ν equals $\lim_{\phi\to 0}(\eta_{sp}/\phi)$[9] in which η_{sp} represents specific viscosity and ϕ relative volume concentration[10]. Hence, the axial ratio can be calculated, if the specific viscosity and relative volume concentration are determined.

Experiment. In the present experiment, the relative viscosity η_r was measured in an Ostwald type viscosimeter of Neurath *et al.*[11] placed in a water-thermostat at 25°±0.02°C. The relative volume concentration was reckoned according to Svedberg & Chirnoaga[12] from the apparent partial specific volume which is obtained in turn by the equation:

$$V=\frac{1}{h}\left(\frac{w}{\rho_d}-\frac{l-h}{\rho_d}\right)$$

where V denotes apparent partial specific volume, w, l and h weights of the solvent, solution (25°C) and solute (dry substance) in a pycnometer respectively, and ρ_d density of the solvent (25°C). w, l, h and ρ_d were 1.8058 g., 1.8155 g., 0.0182 g. and 1.0059 g./m*l.* each to each, and V was calculated at 0.464 m*l.*/g. Consequently, the relative volume concentrations ϕ's of the substance in its solutions are given by multiplying the concentrations in g./d*l.* of the solutions, divided by 10^2, by 0.464. The results are embodied in Table I. In Fig. 2, η_{sp}/ϕ values are plotted against ϕ. The intercept on the ordinate of the straight line, given by application of the least square method, namely, ν was 36.12. The axial ratios were read

TABLE I

η_{sp}/ϕ of Solutions of the Mucopolysaccharide

No. of solutions	C (Concentration in g./d*l.*)	ϕ (Relative volume concentration)	η_r (Relative viscosity)	η_{sp} (Specific viscosity)	η_{sp}/ϕ
1	0.5012	$0.232_5\times10^{-2}$	1.100_3	0.100_3	43.1_4
2	0.2501	$0.116_0\times10^{-2}$	1.047_5	0.047_5	40.9_4
3	0.1251	$0.058_0\times10^{-2}$	1.022_2	0.022_2	38.2_7
4	0.1000	$0.046_4\times10^{-2}$	1.017_5	$0,017_5$	37.7_3

from the curves of f against ν delineated from the figures of Mehl *et al.*[13] and ν of 36.12 above was found to correspond to f of $\frac{19.1}{1}\fallingdotseq\frac{19}{1}$ for rods and to f of $\frac{51.2}{1}\fallingdotseq\frac{51}{1}$ for disks.

Molecular weight calculated from osmotic pressure

Molecular weight can be calculated from osmotic pressure by th equation of van't Hoff:

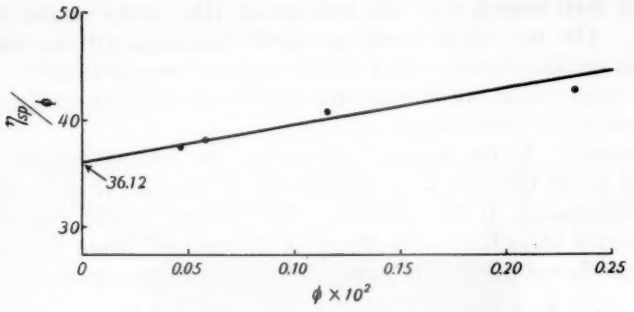

Fig. 2. η_{sp}/ϕ of solutions of the mucopolysaccharide in the phosphate buffer at 25°C as a function of ϕ.

$$M = 2.31_7 \times 10^5 \times (1 + 0.0036_6 t) \frac{C}{p} \quad \cdots\cdots\cdots (2)$$

where M is number-average molecular weight, t temperature (°C), C concentration of a solution in per cent (w/v) and p osmotic pressure. Theoretically C/p is a constant, but practically it incerases when C is raised so that C/p value extrapolated to C of 0.0 must be employed for the calculation.

Experiment. a) Apparatus for measurement of osmotic pressure. An apparatus shown in Fig. 3 was used. The capillary is marked every 1 mm. over the length of 47 cm. The cell contains about 1 m*l*. As the semipermeable membrane was taken a cellophane membrane which had been purified as follows: A commercial membrane was placed in boiling water for 30 minutes at first and then in 5–10% NaOH at 40°C for 15 minutes and after washing with water, preserved in the solvent for preparing the solutions to be examined.

b) Result. The osmotic pressures of variously concentrated solutions at 9°C were measured. It took about 2 weeks to attain equilibrium of the pressures inside and outside the cell. The results are emodied in Table II.

c) Calculation of molecular weight. C/p values were plotted against C and the straight line obtained by the least square method was extrapolated to C of 0.0 (Fig. 4). By substituting C/p in (2) with the intercept on the ordinate (0.66), the molecular weight was calculated at 158,000.

Molecular weight calculated with the axial ratio and diffusion constant

Polson[14] put forward that the relationship expressed in (3) below exists between molecular weight M and diffusion constant D.

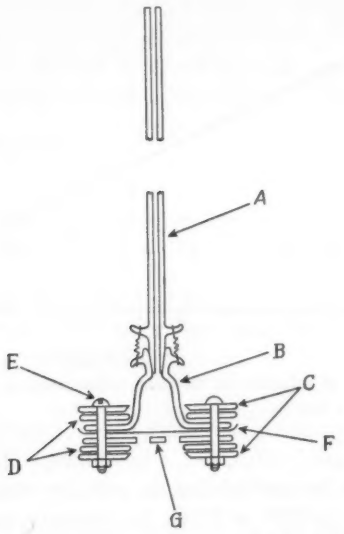

Fig. 3. Diagram of the osmometer. A: capillary (length 600 mm., inner diameter 0.6 mm.), B: cell (content about 1 ml.), C: brass plate gilt with chromium, D: rubber packing, E: screw, F: cellophane membrane, G: porous celluloid plate.

TABLE II

Osmotic Pressure of Solutions of the Mucopolysaccharide at 9°C

No. of solutions	C (Concentration in g./dl.)	p' (Reading in cm.)	ρd (Density)	$p=p'\times\rho d$ (Osmotic pressure in cm. H_2O)	C/p
1	4.92	18.41	1.0307_7	18.97_6	0.25_9
2	3.02	9.94	1.0247_5	10.18_6	0.29_6
3	2.46	6.43	1.0190_4	6.55_2	0.37_5
4	1.51	2.50	1.0179_3	2.54_4	0.59_3

$$M=\frac{(RT)^3}{162\pi^2\eta^3N^2}\cdot\frac{(f_0/f)^3}{D^3V} \quad \cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots \quad (3)$$

where R denotes gas constant, T absolute temperature (°K), η coefficient of viscosity of the solvent, N Avogadro's number, V partial specific volume and f_0/f the reciprocal of Perrin's dissymmetry constant f/f_0, which, according to Perrin[15], equals $\dfrac{\sqrt{1-\rho^2}}{\rho^{\frac{2}{3}}\ln\dfrac{1+\sqrt{1-\rho^2}}{\rho}}$ for rods and $\dfrac{\sqrt{\rho^2-1}}{\rho^{\frac{2}{3}}\tan^{-1}\sqrt{\rho^2-1}}$

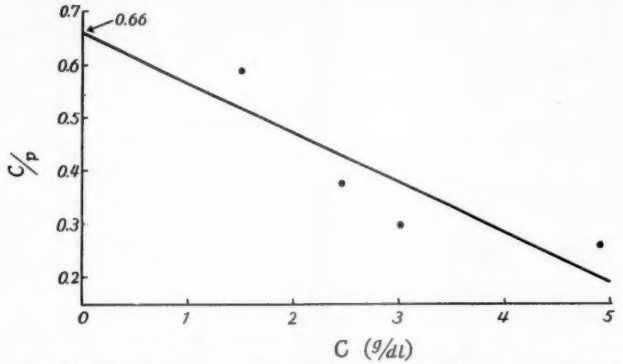

Fig. 4. C/ρ of solutions of the mucopolysaccharide in the phosphate buffer at 9°C as a function of C (g./dl.).

for disks (ρ signifies axial ratio, i.e., f of Simha). In the writer's case, η of the solvent is given by multiplying the relative viscosity of the phosphate buffer of I of 0.147 (1.0358 at 25°C) by viscosity coefficient of water η_0 which amounts to $8.94_9\times10^{-3}$ poise at 25°C. Therefore, its value at 25°C amounts to 9.269×10^{-3} poise, and $\frac{(RT)^3}{162\pi^2\eta^3N^2}$ in (3) comes to 3.35×10^{-14}. Since V has been already measured above, D is the only factor to be determined. This is expressed by the following equation.

$$D=\frac{h}{A}\cdot\frac{Q}{tC} \quad \cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots (4)$$

h is the effective distance through which the solute diffuses, A is the effective area of the pores of the disk, Q is the amount of the material that diffuses in time t, and C is the concentration of the solution.

Experiment. a) Apparatus. A diffusion apparatus whose cell contained 2.87 m*l.* was used. Its ratio $\frac{h}{A}$, named membrane constant or cell constant (K), was determined asccording to Northrop & Anson[16] with 2 *N* NaCl whose diffusion constant is 1.27 cm.^{2}/day or $1.27\times86.4^{-1}\times10^{-3}$ cm.^{2}/sec. 0.198 m*l.* of the saline solution diffused into 5 m*l.* of water in 0.083 day. K was calculated at 0.532 by the relation derived from (4):

$$K=\frac{D\cdot t\cdot C}{Q}=\frac{D\cdot t}{Q/C}$$

where $(Q/C)_{cc}=Q_v$ is the number of cubic centimeters of the concentrated solution that contains an amount of the substance equal to the amount that has diffused.

b) Measurement of D of the mucopolysaccharide. A solution of the

mucopolysaccharide of 1.652 mg./m*l*. in terms of galactose was allowed to diffuse at 25°C against 5 m*l*. of the phosphate buffer. Expressed as galactose, the substance that diffused in 46800 seconds amounted to 0.054 mg. (Masamune and Sakamoto method of analysis[5]). Therefore

$$Q_v=\frac{0.054}{1.652}=0.0327 \text{ m}l.$$

and

$$D'=K.\frac{Q_v}{t}=0.532\times\frac{0.0327}{46800}=3.72\times10^{-7} \text{ cm.}^2/\text{sec.}$$

Here D′ is the constant of diffusion of the solute into the buffer, and was converted into D, constant of diffusion into water at the same temperature, after Polson according to the relation:

$$D=D'\times\eta_r$$

where η_r is relative viscosity of the buffer to water numbering 1.0358 at 25°C. Namely,

$$D=3.72\times10^{-7}\times1.0358=3.85\times10^{-7} \text{ cm.}^2/\text{sec.}$$

c) Calculation of molecular weight. f/f_0 of Perrin is calculated at 1.96 for rods and at 2.39 for disks from the axial ratios given by viscosity measurement. If the reciprocal of those values are replaced for f_0/f in (3), M values of 167,000 $\left(=3.35\times10^{-14}\times\frac{(\frac{1}{1.96})^3}{0.464\times(3.85\times10^{-7})^3}\right)$ and of 92,000 $\left(=3.35\times10^{-14}\times\frac{(\frac{1}{2.39})^3}{0.464\times(3.85\times10^{-7})^3}\right)$ are given for anhydrous prolate and oblate ellipsoids respectively.

Through the Grant Committee for Scientific Researches the Department of Education gave a grant in aid to us.

H. Masamune

References

1) Yosizawa, Tohoku J. Exp. Med., 1949, **51**, 51; 1950, **52**, 111, 145 and 151; 1951, **54**, 115 and 129; 1951, **55**, 35. Masamune & Yosizawa, *ibid.*, 1951, **55**, 43; 1954, **60**, 135. Masamune, Sogo Igaku (Jap.), 1954, **11**, 953.

2) Masamune, Ogawa & Akama, Tohoku J. Exp. Med., 1953, **58**, 234; Masamune, Sogo Igaku (Jap.), 1954, **11**, 953.

3) Yosizawa, Tohoku J. Exp. Med., 1949, **51**, 51.

4) Blix, Acta chemica Scand., 1948, **2**, 467.

5) Masamune & Sakamoto, Tohoku J. Exp. Med., in press.

6) *Idem*, *ibid.*, in press.

7) Masamune, Hakomori, Numabe, Akama & Kamiyama, *ibid.*, in press.

8) Simha, J. Physic. Chem., 1940, **44**, 25.
9) Cf. Bull, Physical biochemistry, 2nd Ed., John Wiley & Sons New York and Chapman & Hall London, 1951, p. 286.
10) Cf. Bull, *ibid.*, p. 284.
11) Neurath, Cooper & Erickson, J. Biol. Chem., 1941, **138**, 411.
12) Svedberg & Chirnoaga, J. Am. Chem. Soc., 1928, **50**, 1401.
13) Mehl, Oncley & Simha, Science, 1940, **92**, 132.
14) Polson, Kolloid Zeits., 1938, **88**, 52.
15) Cited by Polson (14)).
16) Northrop & Anson, J. Gen. Physiol., 1928–29, **12**, 543; 1937, **20**, 575.

Note at proof-reading: The article of Masamune *et al.* (7)) has been published in that Journal, 1955, **61**, 283.

The Tohoku Journal of Experimental Medicine, Vol. 62, No. 1, 1955

Experimental Studies on the Passage of Non-pathogenic Bacteria through the Kidney

By

Masakazu Saheki
(佐伯 正一)

From the Prof. M. Muto's Surgical Clinic, Tohoku University, Sendai

(Received for publication, April 16, 1954)

The question whether bacteria pass through the normal kidney has long been a matter of interests to a great number of investigators, but still remains unsettled even now. Though not a few earlier workers seem to have used experimental methods in which some defects may be pointed out at present, Helmholz and Millikin,[1] Tschiknawerow,[2] Book[3] and others carried out precise experiments, which led them to deny the view that bacteria can pass through the normal kidney. In our country, however there have often been published reports in favor of this view of passage even quite recently (Tsutsui,[4] Nishikawa,[5] Orikasa,[6] Okubo,[7] Abe,[8] and others). As the solution of this question is necessary also from a clinical standpoint, I attempted experiments with non-pathogenic bacteria, in parallel with those using pathogenic bacteria by Mr. K. Monma[9] in our clinic, with the results to deny the passage of bacteria through the kidney and also to explain the reason for the discrepancy of results reported in the literature, as will be described in the present paper.

Experimental

Material and Methods

A total of 42 healthy adult rabbits weighing about 2 kg. were used. Bacteria were injected into the ear veins. Samples of the urine were taken for culture 0.5, 1, 2, 3, 4, 3, 6, and 7 hours after the injection, based upon a common medical knowledge that the excretion by the kidney of foreign bodies in the circulation is at its maximum 0.5 to 1 hour after their introduction into the blood stream.

Bacterial suspension for injection. The species of bacteria used were those of well growing non-pathogenic ones, such as *B. subtilis* (PCI–219), an enterococcus (isolated from normal feces), *B. proteus vulgaris* (OX_{19}), avirulent *B. coli* (divided from the strain kept in the Department of Bacteriology

of our university), and an avirulent acid-fast bacillus *Mycobacterium* 607. For intravenous injection, 5 cc. of 24-hr. broth culture was used in the cases of non-acid-fast bacteria, and 10 mg. of Lockemann's medium culture, suspended in physiological saline solution, was used in the case of *Mycobacterium* 607.

Taking urine samples. In the present investigation, scrupulous care should be taken not to mix blood in collecting the urine. For this, the rabbit was fixed on a bench in dorsal position, the lower abdomen shaved, laparotomy by the lower midline incision performed in anesthetized and sterilized condition, the whole bladder turned out of the incision, and its surroundings covered with a sterile cloth. About 5 cc. of the urine sample was obtained by plunging quickly an 1/3 mm.-needle and syringe into the bladder through a place of the bladder wall where no relatively large blood vessel was present.

Test for bacteria in the urine. Non-acid-fast bacteria were tested for by culture on an ordinary agar plate directly or after having been enriched in broth, and acid-fast bacilli by microscopic examination of the stained sediments of the urine or by culture. For culture, Oka-Katakura's medium was used. The determination of presence or absence of bacteria was made by inspecting 6 tubes for a given sample cultured for 3 months.

Test for bacteria in the blood. A sample of blood was obtained from an ear vein or the femoral vein once before injection of bacterial suspension and at each time of taking the urine (9 samples in all). Non-acid-fast bacteria were tested for by incubating a drop of blood in broth, and acid-fast bacilli by incubating about 2 cc. of blood.

Tests for erythrocytes and albumin in the urine. When a sample of urine was to be obtained, special care was taken in testing for erythrocytes by microscopic examination of the urinary sediments or by the benzidine test. For albumin, the sulfosalicylic acid test was made.

Results

Series 1: *Experiments with B. subtilis.* In each of 6 of 8 rabbits tested, the 8 samples of the bladder urine taken after injection were constantly negative for bacteria both on plate and in broth; they all contained no erythrocytes, and gave negative benzidine test (Table I).

In the remaining 2 cases, on the other hand, the urine was constantly positive in broth in the 1-hr. and later samples, and positive even on plate in the 4- or 6-hr. sample. So far as the results of culture on plate are concerned, the urine had remained negative for bacteria till 3 hours after injection. The positive urine samples contained erythrocytes, increased numbers of erythrocytes being detected in the samples positive for bacteria

TABLE I

Changes by Injection of B. subtilis

No.			Before injection	Time (hrs.) after injection							
				0.5	1	2	3	4	5	6	7
19	Urine culture	in broth	−	−	−	−	−	−	−	−	−
		on plate	−	−	−	−	−	−	−	−	−
	Erythrocytes in urine		−	−	−	−	−	−	−	−	−
	Benzidine test		−	−	−	−	−	−	−	−	−
	Albumine in urine		−	−	−	−	−	−	−	−	−

on plate. The bacterium-positive samples were also positive for the benzidine test, but negative for albumin (Table II).

TABLE II

Changes by Injection of B. subtilis

No.			Before injection	Time (hrs.) after injection							
				0.5	1	2	3	4	5	6	7
17	Urine culture	in broth	−	−	+	+	+	+	+	+	+
		on plate	−	−	−	−	−	−	−	+	+
	Erythrocytes* in urine		−	−	+	++	++	++	++	+++	+++
	Benzidine test		−	−	−	+	+	+	+	+	+
	Albumin in urine		−	−	−	−	−	−	−	−	−

* Erythrocytes; ± Doutful. + Less than one cell in several visual fields. ++ Less than several cells in one visual field. +++ More than several cells in one visual field.

As for bacteria in the blood, the blood constantly contained bacteria throughout the period of examination in each of the 8 cases, even the 7-hr. blood samples revealing numerous colonies upon incubation of only 1 drop of blood in broth and at least several colonies on plate. Accordingly, the concurrence of erythrocytes and bacteria in the 1-hr. and later urine in the 2 cases mentioned suggests that bleeding occurred in the bladder when puncture of the bladder was made twice to take samples and some of the bacteria in the blood stream transferred directly into the bladder.

Series 2: *Experiments with an enterococcus.* In 5 of 8 cases tested with an enterococcus, the urine remained sterile throughout the period ex-

amined in each case.

In the remaining 3 cases (cases 6 to 8), the urine was positive for bacteria in broth culture in the 4-hr. sample of case 6, the 6-hr. sample of case 7, and the 7-hr. sample of case 8, all the other samples being sterile. At about the time of appearance of each positive urine, erythrocytes were detected in the urine and the benzidine test was positive, too (Table III).

TABLE III

Changes by Injection of an Entercococcus

No.			Before injection	Time (hrs.) after injection							
				0.5	1	2	3	4	5	6	7
96	Urine culture	in broth	−	−	−	−	−	−	−	−	+
		on plate	−	−	−	−	−	−	−	−	−
	Erythrocytes in urine		−	−	−	−	+	+	+	+	++
	Benzidine test		−	−	−	−	−	−	+	+	+
	Albumin in urine		−	−	−	−	−	−	−	−	−

As blood incubation tests for bacteria in the present series of experiments constantly yielded positive results as in series 1, even only one drop of the 7-hr. blood revealing bacteria on plate, the presence of bacteria in the 4-hr. or later urine samples of the 3 cases mentioned may be ascribed to the bleeding due to previous punctures of the bladder.

Further, it is to be noted that even in the above 3 cases (where bacteria were detected in the 4-hr. or later urines) the urines were always negative on plate. Thus, so far as the results of culture on plate are concerned, in each case of series 2 the urine remained constantly sterile throughout the period of examination.

Series 3: *Experiments with B. proteus vulgaris.* Of 8 cases tested, only 2 (cases 1 and 2) yielded the results that the urine was negative for bacteria both on plate and in broth throughout the period of examination. In case 3, the urine had been sterile till 2 hours after injection, but was positive after 3 hours in broth and after 6 hours on plate. At about the time when the urine became positive for bacteria there was observed the first appearance of erythrocytes in the urine examined.

In the remaining 5 cases, the urine was positive in broth already in the first sample (taken 0.5-hr. after injection) and also on plate in the 2- or 3-hr. and later samples. If some discrepancy in time is looked over, it may be said in these cases, too, that there was observed a concurrence of bacteria and erythrocytes in the urine; and, as erythrocytes increased in

number, the urine samples became positive for bacteria on plate also, and for the benzidine and albumin tests, too (Table IV).

TABLE IV

Changes by Injection of B. proteus vulgaris

No.			Before injection	Time (hrs.) after injection							
				0.5	1	2	3	4	5	6	7
69	Urine culture	in broth	−	+	+	+	+	+	+	+	+
		on plate	−	−	−	+	+	+	+	+	+
	Erythrocytes in urine		−	±	±	##	##	##	##	##	##
	Benzidine test		−	−	−	+	+	+	+	+	+
	Albumin in urine		−	−	--	−	±	±	+	+	+

If solely the results of culture on plate are referred to in the present series of experiments, it will be seen that the urine was sterile throughout the period of examination in 2 cases, and became positive after 2 hours in 1 case, after 3 hours in 3 cases, after 5 hours in case and after 6 hours in the remaining 1 case.

Series 4: *Experiments with Mycobacterium* 607. For the present series of experiments 10 rabbits were used. Tests of urine for bacteria were made by microscopic examination of the stained sediments of the urine and by cultivation, but the former method was found much inferior to the latter for the present purposes; the results will, therefore, be described here only of cultivation:

In 2 of the 10 cases, the urine remained free from both bacteria and erythrocytes throughout the period of examination.

In 3 cases, the urine had remained sterile for the first 3 hours, but became positive 4 or 5 hours after injection; the positive urines were found to be accompanied by erythrocytes (Table V).

In the remaining 5 cases, the urine had become positive within 30 minutes (1 case), 1 hour (1 case) or 2 hours (3 cases); also in these cases the positiveness of the urine for bacteria was seen to be associated with the presence of erythrocytes or the positiveness of the benzidine test (Table VI).

Series 5: *Experiments with avirulent B. coli.* In 8 cases tested there was not a single case in which the urine remained sterile throughout the period of examination. In only 1 case the ruine had remained sterile in broth for the first 1 hour and on plate for the first 4 hours after injection; erythrocytes were detected in none of the positive urines.

TABLE V

Changes by Injection of Mycobacterium 607

No.		Before injection	Time (hrs.) after injection							
			0.5	1	2	3	4	5	6	7
39	Urine culture	–	–	–	–	–	–	+	+	+
	Erythrocytes in urine	–	–	–	–	±	–	±	+	+
	Benzidine test	–	–	–	–	–	–	–	–	+
	Albumin in urine	–	–	–	–	–	–	–	–	–

TABLE VI

Changes by Injection of Mycobacterium 607

No.		Before injection	Time (hrs.) after injection							
			0.5	1	2	3	4	5	6	7
38	Urine culture	–	–	–	+	+	+	+	+	+
	Erpthrocytes in urine	–	–	–	+	##	##	##	##	##
	Benzidine test	–	–	–	–	+	+	+	±	±
	Albumin in urine	–	–	–	–	–	–	–	–	–

In the remaining 7 cases, all the broth culture tests yielded positive results already with the first samples (the 0.5-hr. urines); in 3 cases of them there was seen a general parallelism between the presence of bacteria and the appearance of erythrocytes in the urine, while in the remaining 4 cases erythrocytes were not demonstrated in some of the bacterium-positive urines, which findings are not easy to explain (Table VII).

TABLE VII

Changes by Injection of Avirulent B. coli

No.			Before injection	Time (hrs.) after injection							
				0.5	1	2	3	4	5	6	7
7	Urine culture	in broth	–	+	+	+	+	+	+	+	+
		on plate	–	–	–	+	–	–	+	+	+
	Erythrocytes in urine		–	–	–	–	–	–	–	±	±
	Benzidine test		–	–	–	–	–	–	–	–	–
	Albumin in urine		–	–	–	–	–	–	–	–	–

So far as the results of culture on plate are concerned, the urine became positive for bacteria within 1 hour in 1 case, 2 hours in 1, 3 hours in 1, 4 hours in 2, 5 hours in 2, and 7 hours in 1.

Series 6: *Experiments with avirulent B. coli on rabbits whose bilateral ureters had been ligated before injection.* In the experiments thus far described, rabbits were intravenously injected with varying kind of bacterial suspension and samples of the bladder urine were taken by puncture of the bladder. In some cases the urine was constantly sterile throughout the period of examination; in the other cases the urine was often found positive for bacteria, where it was generally found that the positiveness of samples for bacteria was seen in association with the presence of erythrocytes, with the exceptions of the cases with avirulent *B. coli*, *B. proteus vulgaris*, and *Mycobacterium* 607, in which there were encountered bacterium positive but erythrocyte-negative urine samples. To clarify the reason for the appearance of these exceptions, the following experiment was made: After preventing urine from flowing into the bladder by bilateral ligature of the ureters, a suspension of avirulent *B. coli* was injected intravenously; then, in order to see whether bacteria made their appearance in the urine that had deposited in the bladder before the ligature, 7 urine samples were taken by puncturing the bladder at intervals of 10 minutes during the period between 10 and 70 minutes after injection. With 5 rabbits tested it was found that bacteria made their appearance already in the first or second sample of the bladder urine; for example, the urine in case 4 (Table VIII) was negative for bacteria in the first sample, but became positive in broth in the second sample and on plate in the fourth (40-min.). In these positive urines erythrocytes were also detected and the benzidine test became positive later. These findings suggest that the appearance of bacteria in the urine was due to bleeding caused by puncture of the bladder.

TABLE VIII*

Changes by Injection of Avirulent B. coli

No.			Before injection	Time (mins.) after injection						
				10	20	30	40	50	60	70
78	Urine culture	in broth	−	−	+	+	+	+	+	+
		on plate	−	−	−	−	+	−	+	+
	Erythrocytes in urine		−	−	±	+	+	+	+	+
	Benzidine test		−	−	−	−	±	+	+	+
	Albumin in urine		−	−	−	−	−	−	−	−

* Case 4, with bilateral ureters ligated.

In case 2 (Table IX), on the other hand, the urine was positive in broth already in the first sample and became positive also on plate in the 30-min. sample, while it was not until the 60- or 70-min. sample was examined that the presence of erythrocytes was clearly demonstrated.

TABLE IX*

Changes by Injection of Avirulent B. coli

No.			Before injection	Time (mins.) after injection						
				10	20	30	40	50	60	70
79	Urine culture	in broth	−	+	+	+	+	+	+	+
		on plate	−	−	−	+	+	−	+	+
	Erythrocytes in urine		−	−	−	−	−	−	++	++
	Benzidine test		−	−	−	−	−	−	+	+
	Albumin in urine		−	−	−	−	−	−	−	−

* Case 2, with bilateral ureters ligated.

Further, it is to be noted that the time of appearance of bacteria on plate in these 5 cases was earlier than in the previous experiments with non-ligature of the ureters, being within 30 minutes in 3 cases, 40 minutes in 1, and 50 minutes in 1, which makes a marked contrast with the time of appearance in the previous experiments: after 4 hours or later in more than half the cases and within 2 hours in only 2 cases out of the 8. This earlier appearance of bacterium-positiveness on plate in the present series of experiments with the same bacterial suspension as in the previous one might conceivably be due to heavier bleeding by frequent puncture of the bladder within a short period of time. The appearance of bacteria in the bladder urine in the present series of experiments, where the ureters had been ligated bilaterally, might not be explained in other way than that bacteria in the blood stream transferred directly into the bladder on account of bleeding due to the bladder puncture. The discrepancy in the time of appearance in the urine between bacteria and erythrocytes may be explained as follows: At the time of previous punctures there occurred bleeding, but it was only slight; and a large majority of erythrocytes sank deep beyond the reach of the needle of a syringe, thus escaping being sucked up together with the bladder urine. A similar discrepancy as seen in series 5 of experiments may be explained in the same manner.

DISCUSSION

In my series of experiments with *B. subtilis* (series 1) and with an

enterococcus (series 2), 6 and 5 out of the 8 cases yielded the results showing the incapability of bacteria to pass through the normal kidney in that the urine in each case was found upon incubation to remain constantly sterile during the period till 7 hours after the intravenous injection of bacteria. In the remaining 2 cases in series 1 and 3 in series 2, the urine was at first sterile but later became positive, although upon culture on plate in thest 3 cases in series 2, the urines were constantly free from bacteria throughout the period of examination.

With the positive urines in series 1 and 2 there were obtained those findings, such as the appearance of erythrocytes and positive benzidine tests, which were suggestive of bleeding in the bladder due to the previous punctures for taking urine samples. In addition, the blood constantly contained bacteria throughout the period of examination in all cases, even the plate culture, as well as the broth culture, of only one drop of the 7-hr. blood sample revealing not less than several colonies. From these facts the occurrence of bacteria in the bladder urine may be taken due to contamination with bacterium-containing blood flown out at the times of previous bladder punctures.

In series 3 of experiments with *B. proteus vulgaris*, the urine was constantly sterile throughout the whole period in 2 out of the 8 cases. In other 1 case, the urine became positive in broth 3 hours after injection and also on plate after 6 hours, which may also be due to the bleeding. In the remaining 5 cases, the urine was positive in broth already at the first test after injection; it became positive on plate, too, in the 2-, 3-, or 5-hr. sample. Erythrocytes were detected in these positive urines, and their time of the first appearance agreed with that of bacteria on plate. The appearance of bacteria in broth, however, was seen earlier than that of erythrocytes.

In series 4 of experiments with *Mycobacterium* 607, 2 out of the 10 cases produced sterile urine throughout the whole period, and 3 cases yielded positive results first after 4 or 5 hours; thus, it will be noted that these 5 cases had produced sterile urine till 3 hours after injection. In the remaining 5 cases. the urine became positive 1 or 2 hours after injection. In this series of experiments the time of appearance of bacteria was found to agree well with that of erythrocytes.

In series 5 of experiments with avirulent *B. coli*, there was observed only 1 case in which the urine had remained sterile in broth for the first 1 hour. In the remaining 7 cases, the urine was constantly positive for bacteria in broth throughout the period of examination, while it became positive on plate 1 to 7 hours after injection; bacteria and erythrocytes did not always appear simultaneously in the urine, the bacterium-positiveness

appearing in advance frequently.

In series 6 of experiments with avirulent *B. coli*, in which the ureters had been ligated bilaterally, before injection, to prevent the renal urine from flowing into the bladder, and urine samples were taken at intervals of 10 minutes during the period of 70 minutes following injection, the urine was positive for bacteria. This positiveness seems to have been due to bleeding at the time of puncture. To the discrepancy in the time of appearance as sometimes observed between bacteria and erythrocytes the following explanation has been given: There occurred a slight bleeding at the time of previous punctures, but a large majority of erythrocytes sank deep beyond the reach of the needle of a syringe and escaped being mixed into the urine sample. In addition, it is to be noted that the earlier appearance of bacteria on plate in this series of experiments than in the other is suggestive of the dependence of the appearance of bacteria in the urine upon the frequency of punctures.

In performing precise experiments on the passage of bacteria through the normal kidney, the most important thing is to obtain urine samples which are free from blood as aseptically as possible. Among various methods devised for obtaining urine samples, the method in which samples are obtained by puncturing the exposed bladder wolud be the best, as pointed out by Helmholz and others,[10] Okubo,[7] and other workers, though it is not without defects. In the present study, too, preliminary tests were made for comparing different methods, and it was found that more accurate results were to be obtained with urin samples taken by direct puncture of the exposed bladder than with samples taken by means of a metal catheter or the Nélaton's catheter, or by puncture through the abdominal wall.

A large number of investigations have been reported as to this problem; and two opposite conclusions have been published: one, in favor of the view of the passage, and the other, against this view. This difference may be due to differences in the amount of intravenous injection of bacteria, in the method for obtaining urine samples, and in other points. Closer inspection of experimental results obtained by various authors, on the other hand, tells us that quite different conclusions may be derived by different interpretations of similar experimental results.

There have been published in our country several papers in support of the view of the passage of bacteria through the normal kidney, such as a paper in which the appearance of bacteria has been demonstrated in the bladder urine already 5 minutes after injection of bacteria into the blood stream (Okubo[7] and Sasaki[11]), a paper in which bacteria have been demonstrated in some samples of the bladder urine taken by means of the Nelaton's catheter after some time following intravenous injection of bac-

teria (Tsutsui[4]), a paper in which there were no direct evidences as to the presence of bacteria in the bladder urine, but conclusions in favor of the view of passage have been reached from histologic examination revealing bacteria in the renal tubules (Tsutsui[4] and Abe[8]), and a paper in which bacteria have been demonstrated in the bladder urine with increase in number of bacteria in the blood stream (Orikasa[6]).

In the present study, where suspensions of five species of well-growing, non-pathogenic bacteria were intravenously injected in great amounts, the bladder urine would often remain constantly sterile in broth as well as on plate even 7 hours after injection when experimental techniques were successful, which results deny the passage of bacteria through the kidney.

It was also often the case with the present study that the bladder urine that had been sterile at first became positive for bacteria 3 to 4 hours after injection, simultaneously with the appearance of erythrocytes. According to Dyke,[12] those bacteria which make their appearance in the bladder urine first after 3 to 4 hours cannot be regarded as the *secretion* or *excretion* of the kidney. From the facts that the excretion from the kidney of pigments, such as phenolsulfonphthalein or methylene blue, or other foreign bodies such as a contrast medium for intravenous pyelography reaches its maximum 30 minutes after the intravenous injection, becoming very small in amount after the intravenous injection, becoming very small in amount after 2 hours, I approve Dyke's opinion. And indeed in the present study it has been demonstrated that the appearance of bacterium-positive bladder urines first after 3 or 4 hours was due to secondary bleeding caused by puncturing the bladder.

Further, it was found in some cases that the appearance of bacteria was in advance of that of erythrocytes. Ane indeed some cases of bilateral ligature of the ureters were encountered in which there occurred bleeding but erythrocytes were not mixed in the urine sample, and they appeared first in later urine samples.

The 5 species of bacteria tested yielded different results; *B. coli*, the most actively multiplying and best growing one among them, was found to appear in the bladder urine earlier than other bacteria. As the puncture of the bladder was made before bacterial injection in this study to confirm the sterility of the bladder urine before injection, there might possibly occur some cases in which this very well growing *B. coli* enter the bladder urine upon bleeding due to puncture aud make later urine samples positive.

As to experiments on the passage of bacteria through the kidney different results have been reported on account of the difficulty encountered in taking the bladder urine free from blood aseptically and periodically. Even if similar results have been obtained, different conclusions may conceivably

be reached in some cases owing to differences in interpretation of the results. The results obtained in the present study also contain both those results which can deny the passage of bacteria completely and those which cannot deny it with certainty. The latter doubtful results, however, were found to have been due to the bleeding caused by puncture of the bladder, and therefore these results are against the view that bacteria can pass through the normal kidney.

Summary

With a view to ascertaining whether bacteria can pass through the normal kidney, suspensions of 5 species of nonpathogenic bacteria were abundantly injected into the ear veins of rabbits, samples of the bladder urine were taken by direct puncture of the previously exposed bladder, and the urine samples were incubated and tested for bacteria, with the results to be summarized as follows:

1. With *B. subtilis* and an enterococcus, the bladder urine constantly remained sterile, in relatively many cases, throughout the period of examination from 0.5 to 7 hours after injection (8 samples). Similar results were obtained in a few cases with other species of bacteria. These results are in support of the view denying the passage of bacteria through the normal kidney.

2. In some cases, the bladder urine was sterile at first but became positive 3 or 4 hours after injection. In the positive urine samples concurrent appearance of erythrocytes was demonstrated. From the common knowledge as to the excretion by the normal kidney of pigment or some other foreign body, those bacteria that appeared in the bladder urine first after 3 or 4 hours cannot be regarded to have been excreted by the kidney, but may possibly be what had transferred from the blood stream into the bladder urine on account of bleeding due to puncture of the bladder.

3. With avirulent *B. coli*, which is the most actively multiplying and best growing species of the bacteria tested, it was often the case that the appearance of bacteria in the urine was in advance of that of erythrocytes. In experiments of bilateral ligature of the ureters (with avirulent *B. coli*) also similar cases were observed when bacteria in the blood stream transferred directly to the bladder urine.

4. These results may be taken to be in favor of the view denying the passage of bacteria through the normal kidney.

References

1) Helmholz & Millikin, Amer. J. Dis. Child., 1925, **29**, 497.
2) Tschiknawerow, Zschr. Hyg., 1933, **114**, 610.

3) Book, Amer. J. Path., 1933, **9**, 569.
4) Tsutsui, Fukuoka Ikadaigaku Zasshi (Jap.), 1927, **20**,125.
5) Nishikawa, Manshu Igaku Zasshi (Jap.), 1934, **21**, 69.
Nishikawa, *ibid.*, 1936, **24**, 301.
6) Orikasa, Nihon Hinyokika Gakkai Zasshi (Jap.), 1936, **25**, 631.
7) Okubo, Hokkaido Igaku Zasshi (Jap.), 1941, **19**, 1062.
8) Abe, Tohoku J. Exp. Med., 1949, **50**, 207.
9) Monma, *ibid.*, in press.
10) Helmholz, Ruth & Field, Amer. J. Dis. Child., 1925, **29**, 641.
11) Sasaki, Hifuka Kiyo (Jap.), 1929, **14**, 487.
12) Dyke, J. Path. Bact., 1923, **26**, 164.

The Tohoku Journal of Experimental Medicine, Vol. 62, No. 1, 1955

Experimental Studies on the Passage of Pathogenic Bacteria through the Kidney

By

Kyoichi Monma
(門馬 杏一)

From the Prof. M. Muto's Surgical Clinic, Tohoku University, Sendai

(Received for publication, April 16, 1954)

With a view to settling the question whether bacteria pass through the normal kidney, I attempted experiments with five strains of pathogenic bacteria, in parallel with those using non-pathogenic bacteria by Mr. Saheki[1] in our clinic, with the results to be reported in the present paper.

Experimental

Material and Methods

The experimental methods used were essentially the same as those employed by Saheki. As test animals a total of 42 healthy adult rabbits weighing about 2 kg. were used. After injecting bacteria into the ear vein of a rabbit, 8 urine samples were taken during the period of 7 hours after injection (0.5, 1, 2, 3, 4, 5, 6 and 7 hours after injection) by puncturing the bladder that had previously been exposed above the pubis.

Bacterial suspension for injection. The following 5 strains of pathogenic bacteria were used: *Staphylococcus aureus* of Heatley strain and of Terashima strain, *B. typhosus* No. 58, *B. pyocyaneus* (divided from the strain kept in the Department of Bacteriology of our university), and *Mycobacterium tuberculosis humanis* of Aoyama B strain. For intravenous injection, 5 cc. of 24-hr. broth culture was used in the cases of non-acid-fast bacteria, and 100 mg. of Lockemann's medium culture, suspended in physiological saline solution, was used in the case of *Mycobacterium tuberculosis*.

Test for bacteria in the urine. B. typhosus was cultivated on Endo's medium directly or after having been enriched in Kauffmann's compound medium. The other non-acid-fast bacteria were cultivated on ordinary agar directly or after having been enriched in broth. Test for *Mycobacterium tuberculosis* was made after cultivation of Oka-Katakura's medium or by microscopic examination of the stained sediments of the urine.

Test for bacteria in the blood. A sample of blood was taken at each time

of obtaining the urine sample (8 samples in all). The presence or absence of bacteria was determined after incubating 1 drop of blood for non-acid-fast bacteria and 2 cc. of blood for *Mycobacterium tuberculosis*.

Tests for erythrocytes and albumin in the urine. Special care was taken for determining the presence or absence of erythrocytes in the urine samples by microscopic examination of the sediments of the urine as well as by the benzidine test. For albumin was performed the sulfosalicylic acid test.

Results

Series 1*: Experiments with Staphylococcus aureus of Heatley strain.* In 3 out of 8 cases tested, the bladder urine remained constantly sterile both on plate and in broth throughout the whole period of examination after injection (Table I).

TABLE I

Changes by Injection of Staphylococcus aureus of Heatley Strain

No.			Before injection	Time (hrs.) after injection							
				0.5	1	2	3	4	5	6	7
1	Urine culture	in broth	–	–	–	–	–	–	–	–	–
		on plate	–	–	–	–	–	–	–	–	–
	Erythrocytes in urine		–	–	–	–	–	–	–	–	–
	Benzidine test		–	–	–	–	–	–	–	–	–
	Albumin in urine		–	–	–	–	–	–	–	–	–

In other 3 cases, the urine had been sterile till 2 hours after injection, but became positive in broth in the 3-hr. sample and also on plate in the 4- or 5-hr. sample (Table II). So far as the results of culture on plate are concerned, the urine had remained sterile till 3 hours after injection. Erythrocytes were demonstrated in these positive urine samples, where the benzidine tests became positive simultaneously with or a little later than the appearance of erythrocytes and the tests for albumin were positive or negative.

In the remaining 2 cases, the urine became positive in broth for bacteria already at the first test (0.5 hour after injection) and also on plate in the 1- or 4-hr. sample. Erythrocytes were detected in these positive urines, where the benzidine tests became also positive a little later than the appearance of erythrocytes. An increased number of erythrocytes were found as the urine became positive on plate (Table III).

TABLE II

Changes by Injection of Staphylococcus aureus of Heatley Strain

No.			Before injection	Time (hrs.) after injection							
				0.5	1	2	3	4	5	6	7
3	Urine culture	in broth	−	−	−	−	+	+	+	+	+
		on plate	−	−	−	−	−	+	+	+	+
	Eryihrocytes* in urine		−	−	−	−	+	++	++	++	++
	Benzidine test		−	−	−	−	+	+	+	+	+
	Albumin in urine		−	−	−	−	−	−	−	−	−

* Erythrocytes; ± Doutful. + Less than one cell in several visual fields. ++ Less than several cells in one visual field. +++ More than several cells in one visual field.

TABLE III

Changes by Injection of Staphylococcus aureus of Heatley Strain

No.			Before injection	Time (hrs.) after injection							
				0.5	1	2	3	4	5	6	7
44	Urine culture	in broth	−	+	+	+	+	+	+	+	+
		on plate	−	−	−	−	−	+	+	+	+
	Erythrocytes in urine		−	±	+	+	++	+++	+++	+++	+++
	Benzidine test		−	−	−	+	+	+	+	+	+
	Albumin in urine		−	−	−	−	−	±	±	±	±

As for the results of incubation of blood, the blood was constantly positive for bacteria throughout the period of examination; incubation of only 1 drop of the 7-hr. sample revealed numerous colonies in broth and at least several colonies on plate. The results that the urine became positive for bacteria in the 0.5- or 3-hr. sample in the 5 cases, therefore, seem to indicate a direct bacterial econtamination of the urine caused by the bleeding in the bladder due to previous 1 to 4 punctures.

Series 2: Experiments with Staphylococcus aureus of Terashima strain. In 4 of 8 cases tested, the urine remained constantly sterile throughout the period of examination.

In other 1 case (case 5 in Table IV), the urine had remained sterile till 5 hours after injection, but became positive in broth in the 6-hr. sample and on plate in the 7-hr. one, where erythrocytes made their first ap-

pearance and the benzidine test was positive.

In the remaining 3 cases, the urine became positive in broth 0.5- or 1-hr. after injection and on plate 3 to 5-hr. after injection. At about the time of appearance of each positive urine, erythrocytes were detected in the urine and the benzidine test was positive, too (Table V).

TABLE IV

Changes by Injection of Staphylococcus aureus of Terashima Strain

No.			Before injection	Time (hrs.) after injection							
				0.5	1	2	3	4	5	6	7
13	Urine culture	in broth	−	−	−	−	−	−	−	+	+
		on plate	−	−	−	−	−	−	−	−	+
	Erythrocytes in urine		−	−	−	−	−	−	−	−	+
	Benzidine test		−	−	−	−	−	−	−	−	+
	Albumin in urine		−	−	−	−	−	−	−	−	−

TABLE V

Changes by Injection of Staphylococcus aureus of Terashima Strain

No.			Before injection	Time (hrs.) after injection							
				0.5	1	2	3	4	5	6	7
49	Urine culture	in broth	−	+	+	+	+	+	+	+	+
		on plate	−	−	−	−	−	+	+	+	+
	Erythrocytes in urine		−	±	±	##	##	##	##	##	##
	Benzidine test		−	−	−	+	+	+	+	+	+
	Albumin in urine		−	−	−	−	−	−	−	−	−

So far as the results of culture on plate are concerned, it will be seen that in all the cases the urine had been sterile for the first 2-hr. after injection.

As incubation on plate of only 1 drop of the 7-hr. blood yielded bacterium-positive results as in series 1 of experiments and as the positive urines were accompanied by erythrocytes, the bacterium-positiveness of the urine seems to have been in association with the bleeding due to previous punctures of the bladder.

Series 3: Experiments with B. typhosus. In 5 of 8 cases tested, the bladder urine remained constantly sterile both on plate and in broth through-

out the period of examination.

In the remaining 3 cases, the urine became positive in broth in the 1-, 3-, or 4-hr. sample and on plate in the 5- or 6-hr. sample; the first appearance of erythrocytes in the urine and the first positive benzidine test occurred in advance of the first appearance of bacteria in the urine (Table VI).

TABLE VI

Changes by Injection of B. typhosus

No.			Before injection	Time (hrs.) after injection							
				0.5	1	2	3	4	5	6	7
84	Urine culture	in broth	−	−	−	−	+	+	+	+	+
		on plate	−	−	−	−	−	−	+	+	+
	Erythrocytes in urine		−	−	+	+	+	+	++	++	++
	Benzidine test		−	−	−	+	+	+	+	+	+
	Albumin in urine		−	−	−	−	−	−	−	−	−

If solely the results of culture on plate are referred to, all the cases had yielded bacterium-negative results for the first 4 hours after injection.

Series 4: Experiments with Mycobacterium tuberculosis humanis of Aoyama B strain. In 8 of 10 cases tested, the urine remained constantly sterile both upon microscopic examination of the sediments of the urine and upon incubation of the urine, though in some instances there were seen contamination of the urine by blood due to frequent punctures of the bladder and there were demonstrated *Mycobacterium tuberculosis* in the blood of such instances, which results might possibly be ascribed to the difficulty of cultivating this species of bacteria.

In case 9 (Table VII), though bleeding occurred already 1 hour after injection, the incubation of the urine for bacteria became positive first in the 7-hr. sample and detection of bacteria by microscopic examination of the stained material was successful only with the 4-hr. urine sample. Similarly in case 10 (Table VIII), the incubation of the urine remained negative throughout the period of examination, but, by the staining method, bacteria were detected in the 5- to 7-hr. samples.

Series 5: Experiments with B. pyocyaneus. In only 1 out of 8 cases tested, the urine was sterile in broth at first, becoming positive already in the 1-hr. sample and remaining so thereafter. In the remaining 7 cases, the urine was constantly positive in broth throughout the period of examination, and positive also on plate in the 3-, 4-, 5- or 6-hr. and later samples.

TABLE VII

Changes by Injection of Mycobacterium tuberculosis humanis

No.		Before injection	Time (hrs.) after injection							
			0.5	1	2	3	4	5	6	7
61	Culture	−	−	−	−	−	−	−	−	+
	Staining	−	−	−	−	−	+	−	−	+
	Erythrocytes in urine	−	−	±	+	+	##	##	##	##
	Benzidine test	−	−	−	±	+	+	+	+	+
	Albumin in urine	−	−	±	+	+	+	+	+	+

TABLE VIII

Changes by Injection of Mycobacterium tuberculosis humanis

No.		Before injection	Time (hrs.) after injection							
			0.5	1	2	3	4	5	6	7
64	Culture	−	−	−	−	−	−	−	−	−
	Staining	−	−	−	−	−	−	+	+	+
	Erythrocytes in urine	−	−	±	±	±	+	+	+	+
	Benzidine test	−	−	−	−	−	−	±	+	+
	Albumin in urine	−	−	−	−	−	−	−	−	−

Erythrocytes were detected and benzidine tests were positive in most of the positive urines, which results are suggestive of contamination of the urine with blood. There were, however, a few cases in which erythrocytes were recognized later than the appearance of samples positive for bacteria in broth (Table IX).

Series 6: *Experiments with B. pyocyaneous on rabbits whose bilateral ureters had been ligated before injection.* Different from the urines obtained in the cases with other bacteria, the urine in the cases with *B. pyocyaneus* became positive in broth relatively shortly after injection and earlier than detection of erythrocytes. To explain these findings the following series of experiments were made: After checking the flow of the renal urine into the bladder by ligating the bilateral ureters, bacteria were injected intravenously: then, in order to see whether bacteria appear in the urine that had deposited in the bladder before the ligation, 7 urine samples were taken by puncturing the bladder at intervals of 10 minutes during the period be-

TABLE IX

Changes by Injection of B. pyocyaneus

No.			Before injection	Time (hrs.) after injection							
				0.5	1	2	3	4	5	6	7
27	Urine culture	in broth	−	+	+	+	+	+	+	+	+
		on plate	−	−	−	−	−	+	+	+	+
	Erythrocytes in urine		−	−	±	+	+	+	+	++	++
	Benzidine test		−	−	−	−	−	+	+	+	+
	Albumin in urine		−	−	−	−	−	−	−	−	±

tween 10 and 70 minutes after injection. In all of 5 cases tested, the bladder urine became positive in broth already at the first examination (as early as 10 minutes after injection) and on plate in the 40- or 50-min. sample (Table X). It will be seen that the time of appearance of positiveness on plate agreed well with that of detection of erythrocytes in the urine.

TABLE X*

Changes by Injection of B. pyocyaneus

No.			Before injection	Time (mins.) after injection						
				10	20	30	40	50	60	70
29	Urine culture	in broth	−	+	+	+	+	+	+	+
		on plate	−	−	−	−	−	+	+	+
	Erythrocyres in urine		−	−	−	±	++	++	##	##
	Benzidine test		−	−	−	−	+	+	+	+
	Albumin in urine		−	−	−	−	−	−	−	−

* Bilateral ureters ligated.

As for the results in broth, however, the bacterium-positiveness appeared earlier than erythrocytes in a few cases. This may be explained as follows: There occurred bleeding due to the puncture of the bladder before injection for taking the sample to test for its sterility, and bacteria in the blood stream transferred directly into the bladder; this first bleeding was only slight and a great majority of erythrocytes mixed in the bladder might sink deep beyond the reach of the needle of a syringe, thus escaping being sucked up together with the bladder urine; on the other hand, the bacteria that had come in the bladder together with blood might have been dis-

tributed therein so diffusely that the bacteria could be demonstrated by the broth culture method.

In addition, a large difference in the time of appearance of bacteria on plate as seen between the present series of experiments and the previous series without ligature of the ureters (40 to 50 minutes in the former, and 3 hours after injection in the latter series) may be explained to be due to differences in the frequency of puncture and the extent of bleeding.

Discussion

The results obtained in the present investigations by bacteriological examination of urine samples taken by puncturing the bladder after varying periods of time following intravenous injection of five strains of pathogenic bacteria may be summarized as follows: First, those results that the bladder urine remained constantly sterile both in broth and on plate throughout the period of examination after injection (from 0.5 to 7 hours after injection) were obatained from 8 of the 1 cases with *Mycobacterium tuberculosis*, from 5 of the 8 with *B. typhosus*, from 4 of the 8 with *Staphylococcus aureus* of Terashima strain, and from 3 of the 8 with that of Heatley strain, namely, from 20 of a total of 34 cases. Many of these sterile urines were free from erythrocytes or signs of bleeding due to puncture; in a few of the cases with *Mycobacterium tuberculosis* a small number of erythrocytes were demonstrated in the sterile urines. That the amount of bacteria used for injection in the present study was sufficient may be understood from the findings that incubation of only one drop (for bacteria other than *Mycobacterium tuberculosis*) or 2 cc. (for *Mycobacterium tuberculosis*) of the 7-hr. blood samples could reveal positive results as to the presence of bacteria. From these results it may be concluded that bacteria did not make their appearance in the bladder even if a sufficient amount of bacteria had been injected intravenously, a conclusion against the view that bacteria can pass through the normal kidney.

Second, those results with bacteria other than *B. pyocyaneus* that the urine was sterile at the first 0.5-hr. sample, but became positive in broth in 1 to 4 hours after injection and on plate in 3 to 7 hours were obtained from 2 cases with *Mycobacterium tuberculosis*, 2 with *Staphylococcus* of Terashima strain, 3 with *B. typhosus*, and 3 with *Staphylococcus* of Heatley strain. Thus, it will be seen that the urine had been sterile for the first 2 hours after injection so far as the results of culture on plate are concerned. In these cases, erythrocytes were first detected in the urine at the time when the urine became positive for bacteria, this bacterium-positiveness therefore being possibly due to a contamination of the urine with bacteria in the blood caused by the bleeding owing to previous punctures. According to

Dyke,[2] who made similar experiments, those bacteria which make their appearance in the bladder urine first after 3 to 4 hours following injection cannot be regarded as the secretion or excretion of the kidney. From the commonly known facts that the excretion from the kidney of pigments, such as phenolsulfonphthalein or methylene blue, or other foreign bodies such as intravenous urographical agents is at its maximum 0.5 to 1 hour after the intravenous injection, I approve Dyke's opinion.

On the other hand, in 2 of each 8 cases with *Staphylococcus aureus* of Terashima and Heatley strains and in 7 of the 8 cases with *B. pyocyaneus*, the urine was already positive in broth at the first examination after injection, later becoming positive also on plate. In these cases, contamination with blood was demonstrated later; the urine had become not infrequently positive in broth before erythrocytes were detected.

In the experiments of bilateral ureteral ligature undertaken to find the explanation of such discrepancies in the time of appearance between bacteria and erythrocytes, which essentially consisted of ligating the bilateral ureters before injection and taking 7 samples of the bladder urine by puncture at intervals of 10 minutes after injection, the urine was already positive in broth at the first examination (10 minutes after injection). In certain cases, however, erythrocytes were not detected in early positive urines but were demonstrated first at later examinations. This may be explained as follows: There occurred bleeding at the lesion of the mucous membrane produced by the puncture before injection; some blood containing bacteria transferred from them blood stream into the bladder, where actively multiplying and well growing bacteria such as *B. pyocyaneus* diffused in the urine and yielded positive results in broth while erythrocytes sank deep and escaped being sucked up together with the urine. In addition, the much earlier appearance of bacteria on plate in this series of experiments than in the other, too, is suggestive of transferrence of bacteria in the blood stream into the bladder urine caused by bleeding due to puncture performed at a much shorter interval of 10 minutes.

The difficulty of experiments concerning the passage of bacteria through the normal kidney lies in the inexistence of an exact method for obtaining urine samples free from blood as aseptically as possible (Helmholz,[3] Okubo,[4] and others). Thus, the method of obtaining urine samples by puncturing the exposed bladder in an area apparently free from blood vessels has been employed since Helmholz. The mucous membrane of the bladder, however, is so rich in blood vessels and so liable to bleed that special care should be taken not to puncture at vascular portions of the bladder. Indeed, in the present study too, not a few cases other than the 42 reported here were discarded on account of copious bleeding.

A large number of experiments have been reported on the passage of bacteria through the normal kidney. There exist, however, mutually exclusive views. This may be due, on one hand, to the inexistence of an ideal method for obtaining urine samples, and, on the other hand, to differences in interpretation of similar results as pointed out by Saheki.

Out of the 34 cases with *Staphylococcus aureus* of Terashima and Heatley strains, *B. typhosus*, and *Mycobacterium tuberculosis*, 20 cases in which bleeding due to puncture could be avoided yielded the results in favor of the view denying the passage of bacteria. As for other cases in which the urine was sterile at first and became positive later, it has been demonstrated that the bacterium-positiveness of the urine was due to bleeding caused by previous puncture. In addition, the findings that certain strains of bacteria including *B. pyocyaneus* were, contrary to the others, apt to appear in the bladder urine are to be interpreted as not indicative of their passability through the normal kidney but as follows: Actively multiplying and well growing bacteria such as *B. pyocyaneus* may easily be demonstrated when they became mixed in the bladder urine, contrary to *Mycobacterium tuberculosis* and others, which are not well growing.

Summary

In order to settle the question whether bacteria can pass through the normal kidney, suspensions of 5 strains of pathogenic bacteria were injected into the ear veins of rabbits, samples of the bladder urine were obtained by direct puncture of the previously exposed bladder, and the urine samples were cultivated to test for bacteria, with the results to be summarized as follows:

1. Those results that the bladder urine remained constantly sterile throughout the period of examination after injection (from 0.5 to 7 hours after injection) were obtained from 8 cases with *Mycobacterium tuberculosis*, from 5 cases with *B. typhosus*, from 4 cases with *Staphylococcus aureus* of Terashima strain, and from 3 cases with that of Heatley strain, namely, from 20 cases out of 34 cases in total, which results are against the view that bacteria can pass through the normal kidney.

2. The bladder urine had been sterile for a short period of time after injection. In some cases they became positive for bacteria later on account of contamination of the urine with blood, which was caused by bleeding due to puncture of the bladder.

3. Most of the cases with *B. pyocyaneus* and only a few of the cases with *Staphylococcus aureus* of Terashima strain yielded the results that bacteria were detected in advance of the appearance of erythrocytes. It has been demonstrated by a series of experiments of ligating the bilateral

ureters that such a phenomenon may be seen with actively multiplying and well growing bacteria.

References

1) Saheki, Tohoku J. Exp. Med., this volume, p. 27.
2) Dyke, J. Path. Bact., 1923, **26**, 164.
3) Helmholz & Millikin, Amer. J. Dis. Child., 1925, **29**, 497.
4) Okubo, Hokkaido Igaku Zasshi (Jap.), 1941, **19**, 1062.

The Tohoku Journal of Experimental Medicine, Vol. 62, No. 1, 1955

Histopathologic Studies of Gastric Ulcer-Carcinoma

I. On the Origin of Gastric Ulcer-Carcinoma

By

Susumu Majima
(間島　進)

From the Prof. M. Muto's Surgical Clinic, Tohoku University, Sendai

(Received for publication, April 16, 1954)

Making histologic examination of many gastrectomy specimens of gastric carcinoma and ulcer kept in our clinic, I found 86 cases of ulcer-carcinoma, including 18 cases in which the ulcer base was free of carcinoma. Close examination of these 18 cases, which had been found to meet the two critical criteria for the diagnosis of ulcer-carcinoma as set up by Hauser,[1] presented some interesting information as to the development of carcinoma from ulcer, as will be reported below.

Results

Macroscopic observations. The lesions, which measured from 0.5×0.5 cm. to 4×4 cm., were found in the corpus ventriculi in 9 of the 18 cases, and at the pars pylorica in the remaining 9 cases. A remarkable radiating fold of the gastric mucosa toward the ulcer was observed in a part of the ulcer in 3 cases, and over the entire margin in the remaining 15 cases. Cross sections of all the specimens revealed that the muscular coat was completely destroyed, their free ends characteristically bent upward into the ulcer margin, and the base of the ulcer was made up of callous omentum tissue.

Microscopic observations. In 16 of the 18 cases, the entire ulcer was divided into 10 to 45 serial blocks; and in 2 cases a great part of the ulcer was divided into 10 to 15 blocks. These blocks were fixed and embedded in paraffin. When necessary, serial sections were made, and stained with hematoxylin and eosin and with Van Gieson's stain. Mallory's connective-tissue stain, mucicarmine, or the Bielschowsky silver method was also used on some sections.

The 18 cases were classified into two groups according to the depth of carcinomatous infiltration at the ulcer margin: (1) the first grade group (11 cases), in which the infiltration did not beyond the mucous membrane, and (2) the second grade group (7 cases), in which the in-

filtration attained to the muscular coat.

(a) The first grade group of ulcer-carcinoma. Case 1 was a callous ulcer in the corpus ventriculi (Fig. 1). A comparatively high extent of

Fig. 1. Ulcer-carcinoma specimen (Case 1) cut open on the greater curvature. The portions surrounded by broken lines represent the areas of early malignant degeneration.

atypical epithelial proliferation was seen almost all along the margin of the ulcer, and at a place of the margin a single layer of regenerative epithelium was found to extend from the margin of the ulcer toward its base (Fig. 2). At three places (A, B, C in Fig. 1) of the ulcer margin, however,

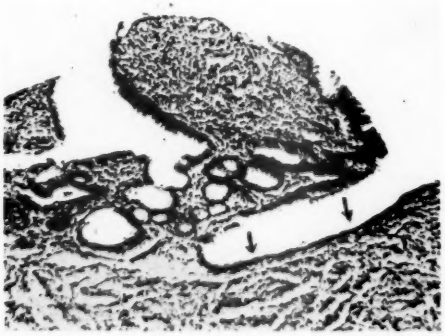

Fig. 2. Part of atypically proliferated epithelium. The arrows point to a layer of regenerative epithelium covering the base of the ulcer.

there were found solitary foci of cancer (i.e., an epithelial overgrowth more advanced than atypical epitherial proliferation). At A, proper gastric glands totally disappeared and were replaced by a highly atypical glandular tissue consisting of acini of various sizes and shapes; the basement membrane of these acini could not be perceived even in silver-

stained sections. The arrangement of cells of the neoplastic glands was irregular and close; the cells were mostly tall cylindrical and partly cuboidal; their nuclei were hyperchromatic, of irregular shape, of various sizes, and showed mitotic figures scatterlingly. These proliferating cells invaded the submucous coat adjoining the ulcer margin to form acini of various sizes and shapes (Fig. 3). B and C were adenomatous cancer

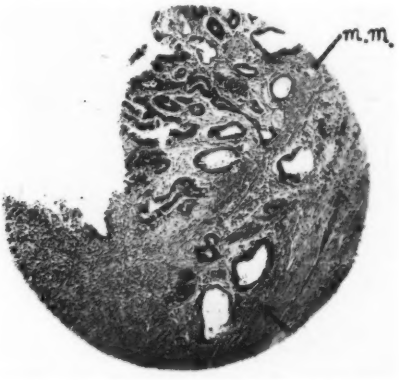

Fig. 3. Area of early malignant degeneration at A of case 1. The arrow points to early cancerous infiltration of the part underneath the muscularis mucosa; m.m., muscularis mucosae.

foci; their glandlike structures were arranged more irregularly than those of A, partly forming solid epithelial cords. However, these foci were localized, there being found no carcinomatous invasion of the submucous coat (Fig. 4). A gradual transition was observed between these three

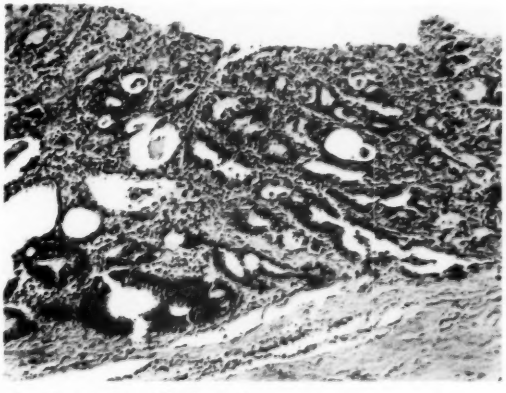

Fig. 4. Cancer focus C of case 1.

cancer foci and the regenerative epithelium of atypical growth.

The mode of development of the cancer focus in the remaining 10 cases belonging to the first grade group is diagramatically shown in Fig. 5. In cases 2 and 3, atypical overgrowth of regenerative epithelium was seen all along the ulcer margin; areas of early malignant degeneration were also noticed at two or three places. In case 2 there were seen, in addition, a small adenomatous cancer focus, which was located 2 cm. apart from the ulcer margin and localized at the mucous membrane.

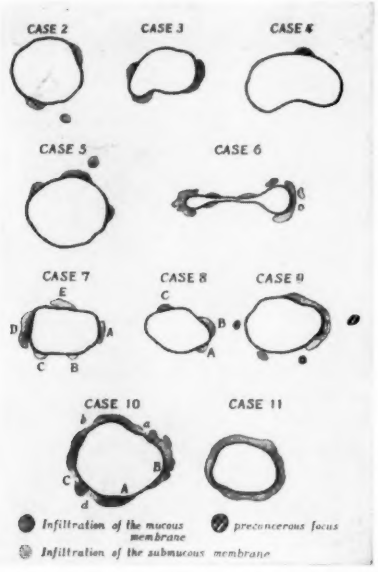

Fig. 5. Diagrams showing the modes of development of ulcer-carcinoma in cases 2 to 11 belonging to the first grade group.

In cases 4 and 5, atypical epithelial proliferation was found in a part of the ulcer margin; areas of early malignant degeneration were also noted at one to three places of the margin. Though they were adenocarcinomas on the whole, the cells were smaller than those in the above three cases and showed a strong tendency to form a solid clump of cells. Partly, carcinomatous infiltration of the submucosa was also observed (Fig. 6). In case 5, a small area of adenocarcinoma was found in addition, 2.5 cm. apart from the ulcer margin. This focus was localized at the mucous membrane on the whole, but carcinomatous invasion of the submucosa was revealed, though only partly.

In case 6, the ulcer was narrow and long, and beared 8 distinct areas

of carcinoma solidum (from $550 \times 1000\ \mu$ to 1.0×1.3 cm.) : 2 comparatively large and 6 small cancer foci. All these were still localized at superficial layers of the mucous membrane, and atypical overgrowth of regenerative epithelium was also seen around them (Fig. 7).

Fig. 6

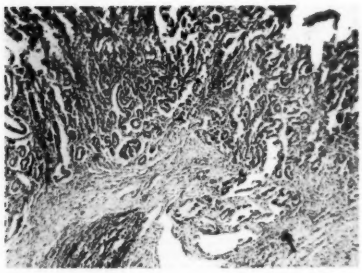

Fig. 7

Fig. 6. Area of early malignant degeneration (Case 5). The arrow indicates infiltration of the submucosa.

Fig. 7. Cancer focus at the ulcer margin (Case 6). A, regenerative epithelium covering the base of the ulcer ; B, base of the ulcer. The portion surrounded by arrows represents the focus of carcinoma solidum.

In cases 7 and 8, 8 cancer foci were observed : 7 foci of carcinoma solidum as in case 6 and 1 focus of tall cylindrical-celled adenocarcinoma (C in case 8, Fig. 8). Many of them were superficial and localized carcinomas of the mucous membrane, while submucous infiltration by cancer cells was noticed at D of case 7 and at C of case 8.

In case 9, which has already been reported in detail by Abe and others,[2)3)] there were found at the ulcer margin 2 solitary adenomatous cancer foci localized between the superficial layer of the mucous membrane and the submucosa, together with 3 solitary precancerous foci.

In case 10, a carcinoma solidum was found almost completely around the ulcer margin, and in case 11 a adenocarcinoma was seen completely around the margin. In case 10, proper gastric glands still remained at the places, a, b, c, and d, where cancer cells existed only interstitially (Fig. 9). This produced the appearance that independent cancer foci had fused at the four places. The cancer foci were mostly confined to the superficial layer ; submucous infiltration, however, was noted at the

Fig. 8

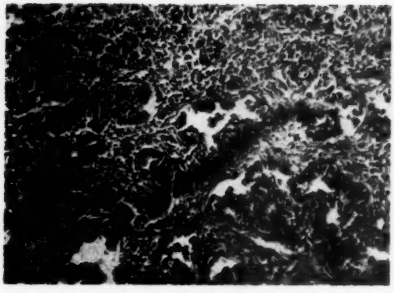

Fig. 9

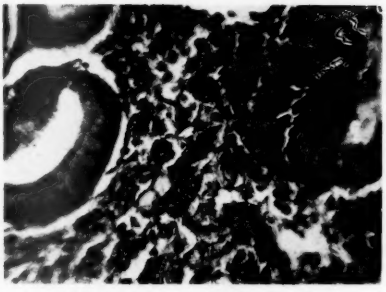

Fig. 8. Cancer focus of tall cylindrical-celled adenocarcinoma (C in case 8)

Fig. 9. Cancerous infiltration as seen at *c* in case 10 in Fig. 5. Only a small number of cancer cells are seen at the area of the interstitial connective tissue.

places A and B in case 10.

(b) The second grade group of ulcer-carcinoma. The mode of development of carcinoma in 7 cases belonging to the second grade group is shown diagramatically in Fig. 10. Of these 7 cases, 3 (cases 12 to 14)

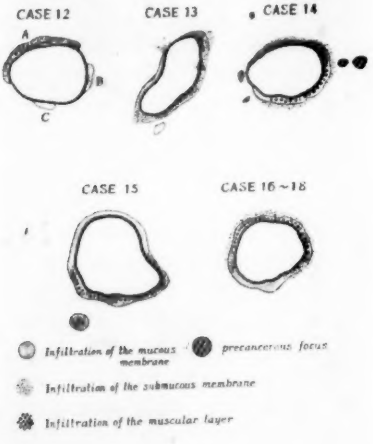

Fig. 10. Diagrams showing the modes of development of ulcer-carcinoma in cases 12 to 18 belonging to the second grade group.

have already been reported in detail by Abe and others.[2)3)] In case 12,

there were found 3 isolated foci: A of adenocarcinoma with cancerous infiltration reaching the muscular layers, and B and C of carcinoma solidum well localized at the mucous membrane of the ulcer margin.

In cases 13 and 14, colloid carcinoma was seen completely around the ulcer margin except a small portion. In addition, a solitary small carcinoma solidum was found in case 13, localized at the mucous membrane and a little apart from the ulcer margin; in case 14, there existed 3 solitary precancerous foci.

In case 15, there were found a carcinoma solidum completely around the ulcer margin and a solitary adenocarcinoma adjoining the pyloric ring and consisting of tall cylindrical cells; part of cancer cells were found to have penetrated the submucosa, attaining even to the muscular layers.

In cases 16 to 18, diffusely infiltrating carcinoma solidum was seen completely around the ulcer margin as in case 15, but the base of the ulcer was entirely free of cancer.

Discussion

Hauser stated that cancerous degeneration in gastric ulcer originates in atypical epithelial proliferation of glandular tissue at the ulcer margin. Moszkowicz[4] sought for the cause of such an atypical proliferation in the reparative function of the epithelium of the gastric foveola. Recently Kuru[5] has reported that ulcer-carcinoma develops in the mucous membrane regenerated for repairing the ulcerated defect. Murakami[6][7] has reported those cases in which glandular and solid cancers developed from regenerative epithelium.

All the areas of early malignant degeneration as found in cases 1 to 8 of the 18 cases in the present study resulted from changes that had occurred in a part of the atypically proliferated mucous membrane at the ulcer margin. Furthermore, areas of early malignant degeneration were clearly recognized in a field of atypical proliferation of regenerative epithelium in cases 1 to 3, and 6. In view of a gradual transition seen between cancer tissue and regenerative mucous membrane in 3 of the above cases, it may be concluded that these areas of early malignant degeneration originated in the regenerative epithelium. From these findings it may be inferred that atypically proliferating cells appear in process of the epithelial regeneration in ulcer and some of them make cancerous change.

On close examination of early gastric cancers as to their development, Konjetzny,[8] Ewing,[9] and others found many early superficial carcinomas over a comparatively large area of the mucous membrane, and asserted that gastric cancer develops multicentrically. Recently, Collins and Gall[10] have supported this conception of multicentric development by

their findings that even with cases of established invasive carcinoma close examination in the neighborhood of the main cancer focus revealed the presence of microscopic pre-invasive carcinoma there, and that even the main cancer focus was often composed of severl cancers of different histologic types. In the case of ulcer-carcinoma also, Hauser,[1] Stoerk,[11] and others stated that cancerous change at the ulcer margin may occur multicentrically at several places of the ulcer margin. Klein,[12] Suzuki,[13] Murakami[6][7] and Ayabe[14] have reported those cases in which cancer developed multicentrically at or around the ulcer margin.

In 7 of the 18 cases (cases 1 to 3, and 5 to 8) in the present study, a total of 29 solitary areas of early malignant degeneration or of small cancer (2 to 8 areas in a case) were found at or apart from the ulcer margin, in 4 cases (cases 9, 12 to 13, and 15), a total of 5 solitary small cancer foci were found (1 to 2 areas in a case), in addition to the main cancer foci occupying a large area at the ulcer margin. The solitude of each of these 34 small " solitary " cancer foci was ascertained by examination of serial blocks of the entire ulcer and of serial sections of part of the ulcer. Twenty-five of these 34 cancer foci were completely restricted to the superficial layer of the mucous membrane; in 8 cancer foci, cancer cells penetrated the muscularis mucosae and reached the underlying submucosa; and only in the remaining 1 focus, cancer cells were found to infiltrate as far as the muscular layers. In each of cases 9 and 14, 3 precancerous foci, independent of cancer foci at the ulcer margin, were also present at the mucous membrane apart from the latter foci. It is also to be noted that solitary cancer foci as found at the ulcer margin or at the mucous membrane apart from it (cases 8, 12, 13, 15) were clearly of different histologic types.

From what has been stated above it may be taken that multiple development of solitary cancer foci has been demonstrated in 11 of the 18 cases. In addition, the following facts are also suggestive of this multiple development: Some of these solitary cancer foci were of different histologic types, and a few of them were at different stages as judged by the extent of infiltration of deeper layers.

In 2 of the 11 cases belonging to the first grade group (where the cancer foci were superficial) and in 6 of the 7 cases of the second grade group (where cancerous infiltration reached the muscular layers) cancer cells were found to have extended completely or almost completely around the ulcer margin. Thus, there was no conclusive evidence of the multicentric development in these cases. However, in 1 case (case 10) of the first grade group pictures indicative of a fusion of independently developed cancer foci were seen; and in 3 cases (cases 13 to 15) there were found small cancerous or precancerous foci, apart from the cancer foci com-

pletely around the ulcer margin and localized at the mucous membrane. From these findings and the incidence of not a few cases demonstrating multicentric development of cancer foci as stated above it may be inferred that even in cases in which a cancer focus completely surrounded the ulcer margin, canceration had originated multicentrically, and independently formed cancer foci fused by their progressive enlargement, with one another.

Summary

1. Examination was made of 18 cases with ulcer-carcinoma in early stages by the serial section method.

2. Ulcer-carcinoma seems to originate from regenerative epithelium of atypical proliferation.

3. In 11 of the 18 cases with early ulcer-carcinoma, multicentric development of cancer foci has been demonstrated at the ulcer margin or in the mucous membrane apart from the ulcer margin. In the remaining 6 cases, except 1 case in which a small cancer focus was found in a very small portion of the ulcer margin, cancer foci extended to surround the ulcer margin completely or almost completely, being thus suggestive of the fusion of multicentrically developed cancer foci.

References

1) Hauser, Handb. spez. path. Anat. u. Hist. (Henke u. Lubarsch), Berlin, 1926, **4**, 497–518.
2) Abe, Majima & Kurakake, Nihon Geka Gakkai Zasshi (Jap.), 1952, **53**, 501.
3) Abe, Majima & Kurakake, Igaku (Jap.), 1952, **8**, 280.
4) Moszkowicz, Arch. klin. Chir., 1924, **132**, 558.
5) Kuru, Nihon Geka Gakkai Zassi (Jap.), 1952, **53**, 537.
6) Murakami, *ibid.*, 1951, **51**, 651.
7) Murakami, Acta Path. Jap., 1952, **2**, 10.
8) Konjetzny, Der Magenkrebs, Ferdinand Enke, Stuttgart, 1938.
9) Ewing, Amer. J. Surg., 1936, **31**, 204.
10) Collins & Gall, Cancer, 1952, **5**, 62.
11) Stoerk, Wien. klin. Wschr., 1925, **38**, 347.
12) Klein, Arch. Surg., 1938, **37**, 155.
13) Suzuki, Mitt. allg. Path., Sendai, 1941, **10**, 219.
14) Ayabe, Igaku (Jap.), 1949, **6**, 268.

The Tohoku Journal of Experimental Medicine, Vol. 62, No. 1, 1955

Histopathologic Studies of Gastric Ulcer-Carcinoma

II. On the Growth of Ulcer-Carcinoma

By

Susumu Majima
(間島 進)

From the Prof. M. Muto's Surgical Clinic, Tohoku University, Sendai

(Received for publication, April 16, 1954)

In the first paper of this series[1] a report was made of 18 cases with early ulcer-carcinoma, in which the focus of cancer had been localized at the ulcer margin. These cases were found on the whole to meet the critical criteria for the diagnosis of ulcer-carcinoma as set up by Hauser.[2] Besides these, 68 cases were obtained in which cancer tissue was found completely or almost completely around the ulcer margin, carcinomatous infiltration extending to a small or great part of the ulcer base or further to the free great or lesser omenta, thus indicative of peritoneal carcinomatosis. To determine these 68 cases to be of ulcer-carcinoma it is necessary to demonstrate that they are not cases of ulcerated carcinoma, or primary carcinoma which has undergone peptic ulceration (Borrmann,[3] Stromeyer,[4] S. H. Klein,[5] Mallory,[6] and others). Since Hauser's and Stromeyer's papers these have been much argument about the judgement of ulcer-carcinoma even in earlier stages. As has been pointed out by Finsterer[7] and others, however, the fact that malignant degeneration of chronic peptic ulcer is possible cannot be denied, aud the point about which discussion still centers seems to be the incidence of such a lesion. The wide variation in the frequency of ulcer-carcinoma as reported in the literature depends partly on the strictness of the criteria adopted for its diagnosis. Severe criteria were adopted by Stromeyer, Borrmann, S. H. Klein, Mallory and others, and moderate criteria by Stoerk,[8] E. Klein and Demuth,[9] Ayabe,[10] Fukuda,[11] Kuru,[12] and others. Aschoff[13] at first stressed the rarity of cancers arising from gastric ulcers, but later he changed his opinion as follows: When carcinomatous proliferation is seen in the area of the submucosa at most, and, macroscopically, characteristic pictures of callous ulcer are presented, there is no room for doubt that the case is of ulcer-carcinoma; in advanced stages of cancer, the diagnosis of ulcer-carcinoma is difficult; by referring to the fact that in chronic peptic ulcer the muscular

layer is destroyed over a wide range, however, the case may safely be determined to be of ulcer-carcinoma when there are seen, at the base with carcinomatous infiltration, a wide space devoid of the muscular layer, and other characteristic features of chronic ulcer are presented.

I too have taken my stand on the wide interpretation of ulcer-carcinoma as Aschoff[13] and others, and have adopted the following criteria for the diagnosis of ulcer-carcinoma: complete destruction of the muscular layer, upward bending of the free ends of the muscular layer into the ulcer margin, inward pulling of the muscularis mucosae to the muscular layer, composition of the base with cicatrized omentum tissue, and other features characteristic of callous ulcer.

As for the state of cancerous infiltration of the ulcer base in our 68 cases, there was found no cancer focus or only a few foci, if any, in the superficial part, whereas the infiltration was remarkable in the deeper part. From these findings it is hardly conceivable that an ulcer had developed secondarily in a primary gastric cancer. As controls, a large number of specimens of primary gastric cancer which disintegrated secondarily were examined microscopically, with the results that lesions in these specimens were quite different from those of ulcer-carcinoma in that the muscle fibers still remained in spite of a remarkable cancerous infiltration of the muscular layers. Clinically too, there were not a few cases in which a long standing ulcer history of 5 to 25 years were proved.

The methods for examining early ulcer-carcinoma were stated in the first report. With the 68 cases of advanced ulcer-carcinoma, 6 to 15 blocks from different parts of an ulcer were taken, and sections were prepared for histologic examination. Characteristic pictures of ulcer obtained with the 86 cases were as follows: The radiating folds of the gastric mucosa toward the ulcer were clearly visible by the naked eye completely around the ulcer margin (42 cases) or in some parts of it (37 cases) with the exception of 7 cases in which no clear radiating fold was visible. Destruction of the muscular layer and turning up of its free ends into the ulcer margin were distinctly seen in all the cases. Pictures of inward pulling of the free ends of the muscularis mucosae were conspicuous in 60 cases, and partly or entirely obscure on account of carcinomatous infiltration in 23 or 3 cases, respectively.

With the exception of 4 cases of penetrating ulcer, these cases were all of callous ulcer, the base of the ulcer being made up of callous omenta.

Incidence of ulcer-carcinoma: The 86 cases of ulcer-carcinoma consisted of 32 cases in which operation had been performed for "gastric ulcer" and 54 cases for "gastric cancer." These cases were selected out of 1045 gastrectomy cases of gastric cancer and ulcer, which were found on histopathologic examination to consist of 698 gastric cancer, 86 ulcer-carcinoma,

and 261 gastric ulcer cases. Thus, the incidence of ulcer-carcinoma in gastric cancer was 10.9 per cent, and 24.9 per cent in gastric ulcer. The data reported by several authors in our country are listed in Table I for

TABLE I

Incidence of Ulcer-Carcinoma in Japan

Author	Clinic	Incidence of ulcer-carcinoma	
		in ulcer	in cancer
Fukuda[11]	Surgical clinic of Prof. Ootuki, Tokyo University	33.3	19.7
Ayabe[10]	Surgical clinic of Prof. Tomoda, Kyushu University	23.8	11.1
Tomoda[15]	Surgical clinic of Prof. Goto, Kyushu University	19.3	4.6
Suzuki[16]	Surgical clinic of Prof. Sugimura, Tohoku University	18.5	9.9
Miyake[17]	Surgical clinic of Prof. Miyake, Kyushu University	16.6	6.6

comparison. According to Newcomb,[14] who reviewed the reports of 102 authors, in about half of the reports the incidence of ulcer-carcinoma in gastric cancer was above 10 per cent, and in the other half below 10 per cent. The variation in the incidence of ulcer-carcinoma as seen in the literature probably depends on the criteria adopted for the diagnosis as well as on the type of cases selected for study.

As for the site of the 86 cases of ulcer-carcinoma, 47 cases were found in the pars pylorica and 39 in the corpus ventriculi. The ulcer measured in maximum diameter 0.1 to 1.9 cm. in 5 cases, 2 to 3.9 cm. in 30 cases, 4 to 5.9 cm. in 37 cases, and 6 to 7.9 cm. in 14 cases.

Extent of surface growth and depth of infiltration of ulcer-carcinoma: With a view to clarifying the correlation of the depth of infiltration with the extent of surface growth of ulcer-carcinoma, Table II was made up, in which the 86 cases were classified in 4 groups according to the depth of infiltration: the group of grade 1, in which the cancer focus was localized at the mucous membrane; of grade 2, in which infiltration extended to the muscular layer; of grade 3, in which infiltration extended to the subserous layer; and of grade 4, in which infiltration extended beyond the serous membrane. As shown in Table II, the groups of grades 1 and 2 of infiltration included 18 cases, which consisted, as reported in the previous paper, of 1 case in which a cancer focus was confined to only a small part of the ulcer margin, 9 cases in which 2 to 8 small solitary multicentric cancer foci were observed at the ulcer margin and its environs, and 8 cases

TABLE II

Degree of Carcinomatous Infiltration of Ulcer Margin and Base

Grade of deeper infiltration	Location of lesion		
	Margin only	Margin and part of base	Margin and large part of base
Grade 1	11		
Grade 2	7		
Grade 3		9	
Grade 4		29	30

in which cancerous infiltration were seen completely or almost completely around the ulcer margin. The cancer foci in mucous membrane at the ulcer margin measured in maximum width 10 mm. in 2 cases, 5 to 9 mm. in 5 cases, and less than 5 mm. in the remaining 11 cases. The range of infiltration of deeper parts was narrow: In the large majority (16 cases) of the cases, cancerous infiltration was restricted to only small area of the submucosa or the muscular layer underneath the ulcer margin, the range of infiltration being relatively wide in only 2 cases of grade 2. It is to be noted that the ulcer base was entirely free from carcinoma in these 18 cases (Figs. 1 and 2).

The grade 3 group, in which carcinomatous infiltration of the sub-

Fig. 1

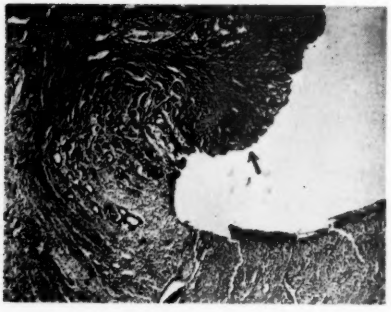

Fig. 2

Fig. 1. An ulcer-carcinoma of grade 1. The arrows point to the cancer focus at the ulcer margin.

Fig. 2. An ulcer-carcinoma of grade 2. The arrow *a* indicates the cancer focus in the mucous membrane at the ulcer margin, and arrow *b* points to the cancer focus at the free end of the destroyed muscular layer. B, base of ulcer.

serous layer was seen, included 9 cases (Table II), where cancer tissue was found to have extended all around the ulcer margin, carcinomatous infiltration being detected in a part of the base. In 5 of these cases, cancer foci at the ulcer margin measured 5 to 6 mm. in maximum width, being nearly as wide as the range of the infiltration at the base; in the remaining 4 cases, cancer foci at the ulcer margin measured 9 to 12 mm. in maximum width, being wider than the infiltration at the base. Infiltration of the base was more marked in the deeper part than in the superficial part, and conspicuous groups of cancer cells were found interstitially in upward bent muscle fibers and callous tissue and seen further in the subserous layer (Fig. 3).

Fig. 3. Ulcer-carcinoma of grade 3. Broken lines indicate the border between the callously thickened subserous layer and the callous omentum composing the base of callous ulcer.

Cases of grade 4 (i.e., with cancerous infiltration extending beyond the serous membrane) were very many in our study, amounting to 59. In these, cancer tissue was found all around the ulcer margin; carcinomatous infiltration of the base was restricted to a small area in 29 cases, and in 30 cases become extended to a large part of the base.

Though cancer tissue completely encircled the ulcer margin as mentioned above, its maximum width was less than 10 mm. in 37 cases; the area of carcinomatous infiltration of the base was of about the same as that of the cancer focus at the ulcer margin in 9 of the cases, larger in 23 cases, and smaller in only 5 cases. There were found 16 cases in which

cancer foci in the mucous membrane at the ulcer margin were 11 to 19 mm. in maximum width; the area of carcinomatous infiltration of the base was of the same size as or larger than that of the ulcer margin in 10 cases, and in 6 cases smaller. Those cases in which cancer tissue completely encircling the ulcer margin measured in maximum width more than 20 mm. amounted to 6, where the cancer foci at the ulcer margin were larger than the area of infiltration of the base. The degree of carcinomatous infiltration of the base was found to increase with the depth. As these ulcer bases were made up of callous omentum tissue, cases with marked infiltration of the base might also be taken to be of early peritoneal carcinomatosis. Indeed, in 15 of the cases of grade 4 a small number of cancer cells were found in the part bordering callous and normal omenta, in normal omentum tissue, or in that portion of liver or pancreas which adhered to the ulcerated part; in the remaining 44 cases carcinomatous infiltration was clearly seen in the normal omentum gathering in the ulcerated part (32 cases) or in the free portion of omentum apart from the ulcerated part (12 cases), as shown in Figs. 4 and 5.

Fig. 4

Fig. 5

Fig. 4. Ulcer-carcinoma of grade 4, in which carcinomatous infiltration of the base was restricted to a small area. Arciform carcinomatous infiltration of the deeper part of the base from the subserous layer is shown by arrows.

Fig. 5. Ulcer-carcinoma of grade 4, in which carcinomatous infiltration of the base extended to a large part of the base. Though a great part of the ulcer base has been infiltrated, there is seen no infiltration of the superficial part of the middle portion of the base was shown by arrows; the base is made up merely of callous tissue. M, upward turned destroyed muscular layer; A, obliterated endoarteritic vessel.

The cancer foci in the 18 cases of grades 1 and 2 of deeper infiltration were all less than 1.0 cm. in maximum width; the multicentric development of each of these foci have been demonstrated or inferred in the first report. In the 9 cases of grade 3 (infiltration penetrating the muscular layer and reaching the subserous membrane) the cancer focus was found to encircle the ulcer margin almost completely; the maximum width of a focus was less than 2.0 cm. (in 6 cases, less than 1.0 cm.). In the 59 cases of grade 4, where carcinomatous infiltration was noticed beyond the serous membrane and in omentum tissue, and a cancer focus completely around the ulcer, the maximum width of most cancer foci was less than 2.0 cm., being more than 2.0 cm. in only 6 cases. These findings too are suggestive of multicentric development of cancer. In the cases of grades 3 and 4 it was difficult to determine whether the cancer tissue had been formed multicentrically or unicentrically, for it encircled the ulcer margin completely; however, it is quite inconceivable that cancer tissue which was monocentric and surrounded completely the periphery of the ulcer, the circumference of which was more than 2.0 cm. in many cases, did remain to be mostly less than 2.0 cm. in maximum width.

As for relations between the size of the focus of a cancer, the degree of infiltrating the deeper part, and the area of infiltration of the ulcer base, in the cases where the focus was localized at the mucous membrane of the ulcer margin (grade 1) or extended to the muscular layer (grade 2), there was noticed no carcinomatous infiltration of the base; in the cases (grade 3) where the infiltration extended through the muscular layer to the subserous membrane, partial infiltration of the base was seen, the area of base infiltration being of the same size as or smaller than that of the cancer focus at the ulcer margin; in the cases (grade 4) where carcinomatous infiltration extended beyond the serous membrane, there was found carcinomatous infiltration of some or a great part of the base, the area of the cancer focus at the ulcer margin was smaller than 2 cm., but the area of base infiltration showed a tendency toward becoming larger than that of the former. General survey of these specimens showing various stages of ulcer-carcinoma will furnish a basis for inference as to the process of growth of ulcer-carcinoma.

Process of growth of ulcer-carcinoma: According to Stoerk,[8] cancer cells that have originated in the mucous membrane at the ulcer margin rarely infiltrate the ulcer base directly; in most cases cancer cells first proliferate from the ulcer margin externally downward and reach the subserous layer, then invade from below the cicatrized tissue composing the base, from where they proceed upward along newly-formed blood vessels until they reach the superficial part and infiltrate it. E. Klein and Demuth,[9] who did not obtain findings indicative of multicentric

development of cancer, were of the opinion as follows: Carcinoma occurs in the margin of the ulcer in a single zone; as the tumor can not penetrate into the dense, fibrous ulcer base, the growth takes place around the margin, which offers less resistance; and, after this stage, the carcinoma will grow in nearly the same order as that pointed out by Stoerk.

The interpretation of the findings obtained with our 86 cases as to the growth of ulcer-carcinoma may be stated as follows (Fig. 6): Carcinoma develops in multicentric foci at the ulcer margin; it grows in

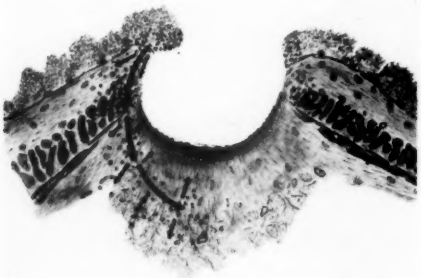

Fig. 6. Diagram showing the process of growth of cancer tissue. The thick arrow indicates the main route, and the thin arrows represent sideward infiltration.

the submucosa independently in each focus, and penetrates the free ends of the muscular layer. On reaching the subserous layer, it proceeds to callous omentum tissue forming the base. There also takes place sideward infiltration from this main route; the infiltration of the superficial part of the ulcer base is due to an inward side infiltration. Simultaneously with this, there is seen not infrequently an outward side infiltration, which extends to the free great and/or lesser omenta or other tissues surrounding the stomach and may give rise to peritoneal carcinomatosis. Even if no carcinomatous infiltration is noticed in the free omentum, a case in which the infiltration has extended to the callous omentum making up the ulcer base is taken, histologically, to be in an early stage of peritoneal carcinomatosis. In ulcer-carcioma, therefore, a bad prognosis is to be given in proportion to the smallness of the area of the carcinomatous infiltration.

Histologic types in ulcer-carcinoma: Histologic types of our 86 cases can be classified as shown in Table III, according to Petersen and Konjetzny. It will be seen that each type of theirs has been found in our cases.

As to the degree of differentiation the following findings were ob-

TABLE III

Histologic Types of 86 Cases of Ulcer-Carcinoma

Histologic type	Number of cases	Percentage
Carcinoma adenomatosum simplex	25	40.7
Carcinoma adenomatosum microcysticum	9	
Carcinoma adenomatosum gelatinosum	1	
Carcinoma solidum alveolare	11	50.0
Carcinoma solidum diffusum	22	
Carcinoma solidum gelatinosum	7	
Carcinoma fibrosum	3	
Mixed form	8	9.3

tained: In many cases adenocarcinoma was made up of well-differentiated cylindrical or cuboid cells; there were found acinic structures in the well-established portion of the tumor. In actively proliferating areas of the periphery around the tumor, clusters or cords of cancer cells were formed, and there was seen a tendency to interstitial infiltration of muscle fibers or callous fibers.

In carcinoma solidum there were mostly found anaplastic small polygonal cells. In the mucous membrane, these cancer cells were relatively poor in stroma, presenting the typical histologic picture of medullary carcioma or a similar picture; but in the submucous, muscular, and subserous layers and dense fibrous tissue forming the base, cancer cells were present diffusely in the interstices of muscular or callous fibers. Thus, most of the cases presented the typical histologic picture of scirrhous carcinoma or a similar picture, there being found only 3 cases in which the whole tumor uniformly presented the picture characteristic of medullary carcinoma.

Kimura[18] has reported that in ulcer-carcinoma there are seen almost all histologic types, and most frequently obtained pictures were such as showing that small cells formed a structure similar to medullary carcinoma, adenocarcinoma also being found not infrequently. Kuru[12] stated that early ulcer-carcinoma in most cases takes, from the beginning, the form of carcinoma simplex of a highly malignant scirrhous carcinoma type though it may take the form of adenocarcinoma microcysticum in some cases. Our 86 cases included 43 cases (50 per cent) of carcinoma solidum, an undifferentiated carcinoma, and 35 cases (41 per cent) of adenocarcinoma, a relatively well differentiated carcinoma; active infiltrating growth was demonstrated in many cases not only of the former form but also of the latter form.

Metastasis of the regional lymph glands in ulcer-carcinoma: As already

mentioned, there were seen not a few cases of ulcer-carcinoma which, despite the smallness of the cancer focus, complicated peritoneal carcinomatosis and are to be given a bad prognosis. Table IV shows the results of examining the regional lymph glands, as classed according to the degree

TABLE IV

Relation of the Depth of Carcinomatous Infiltration with the Metastasis of Lymph Glands

Lymph gland metastasis	Degree of infiltration			
	Grade 1	Grade 2	Grade 3	Grade 4
–	11	4	5	12
+		3	4	47

of infiltration of the deeper part. It will be seen that metastasis of the regional lymph glands was noticed in none of the 11 cases of grade 1 (with cancer tissue localized at the mucous membrane), in 3 of the 7 cases of grade 2 (with carcinoma infiltrating the muscular layer), in 4 of the 9 cases of grade 3, and in 47 (79.7 per cent) of the 59 cases of grade 4.

SUMMARY AND CONCLUSIONS

1. Upon histopathologic examination of 752 cases of gastric cancer and 293 cases of gastric ulcers, 86 cases of ulcer-carcinoma (54 from the former, 32 from the latter cases) were found. Thus, the incidence of ulcer-carcinoma was 24.9 per cent in gastric ulcer, and 10.9 per cent in gastric cancer.

2. A general survey of various stages of ulcer-carcinoma, from early to advanced, with reference to the process of its growth have led us to conclude as follows: Cancer foci, which have developed multicentrically at the ulcer margin extend independently through the submucous layer and the free ends of the muscular layer to the subserous layer, and invade the callous tissue making up the ulcer base. Sideward infiltration of the superficial part of the base and the free omentum tissue also takes place. Therefore, in the stage showing carcinomatous proliferation at the ulcer base, cancer tissue often cause infiltration beyond the serous membrane of the stomach. This is the reason why in ulcer-carcinoma an unfavorable prognosis is to be given for its size.

3. There have been found in our 86 cases each type of histologic pictures as classified by Petersen and Konjetzny.

References

1) Majima, Tohoku J. Exp. Med., this volume, p. 53.

2) Hauser, Handb. spez. path. Anat. u. Hist. (Henke u. Lubarsch), Berlin, 1926, **4**, 497–518.
3) Borrmann, Handb. spez. path. Anat. u. Hist. (Henke u. Lubarsch), Berlin, 1926, **4**, 902–919.
4) Stromeyer, Beitr. path. Anat., Jena, 1912, **54**, 1.
5) Klein, S. H., Arch. Surg., 1938, **37**, 155.
6) Mallory, Arch. Path., 1940, **30**, 348.
7) Finsterer, Proc. Roy. Soc. Med., 1938–39, **32**, 183.
8) Stoerk, Wien. klin. Wschr., 1925, **38**, 347.
9) Klein, E. & Demuth, Beitr. path. Anat., Jena, 1928, **79**, 117.
10) Ayabe, Igaku (Jap.), 1949, **6**, 210.
11) Fukuda, Tokyo Igakkwai Zassi (Jap.), 1943, **57**, 1.
12) Kuru, Nihon Geka Gakkai Zassi (Jap.), 1952, **53**, 537.
13) Aschoff, Schweiz. med. Wschr., 1939, **20**, 261.
14) Newcomb, Brit. J. Surg., 1932–33, **20**, 279.
15) Tomoda, I-Junishicho-Kaiyo (Jap.), Kanehara, Tokyo, 1939.
16) Suzuki, Mitt. allg. Path., Sendai, 1941, **10**, 219.
17) Miyake, Igan (Jap.), Kokuseido, Tokyo, 1928.
18) Kimura, Nippon Shokakibyogakkai Zassi (Jap.), 1929, **28**, 587.

The Tohoku Journal of Experimental of Medicine, Vol. 62, No. 1, 1955

On the Postoperative Prognosis in Ulcer-Carcinoma of the Stomach

By

S. Majima, J. Katami, N. Kurakake and **N. Hoshi**
(間島　進) (片見準一) (鞍掛誠秀)　(星　信男)

From the Prof. M. Muto's Surgical Clinic, Tohoku University, Sendai

(Received for publication, April 16, 1954)

From the findings that a cancer focus in the mucous membrane at the ulcer margin may not infrequently infiltrate the deeper part of the ulcer base though its area is smaller than 2 cm. in maximum width, and that the base of callous ulcer is made up of callous omentum tissue, Majima[1] has concluded, in his paper on the process of development of ulcer-carcinoma, that carcinomatous infiltration of the ulcer base is indicative of an early stage of peritoneal carcinomatosis, and that there are found not a few cases in which only poor prognosis is to be given in spite of the small size of the lesion.

We have made an investigation of late results of gastrectomy in cases in our clinic, and comparison has been made between these results and histopathologic findings with the resected specimens, with the results to be reported in the present communication.

Late Results of Gastrectomy

An investigation has been made of the postoperative course in 70 cases, excluding cases of hospital death and those within one year after operation; and in 60 cases of them late results of gastrectomy became clear, as summarized in Fig. 1, in which the cases have been classed in 4 groups according to the depth of carcinomatous infiltration (Majima),[1] this depth of the infiltration being taken as representative of the histologic findings.

In 10 cases of grade 1, in which cancer tissue was localized in the mucous membrane at the ulcer margin, there has been no case of relapse of disease to death; all the patients are healthy at present, after postoperative 2 to 3 years in 5 cases and 4 to 7 years in 5 cases. Of the 10 cases, 9 were operated on for "gastric ulcer," and 1 for suspicion of malignant degeneration; in no cases was recognized metastasis of the regional lymph nodes.

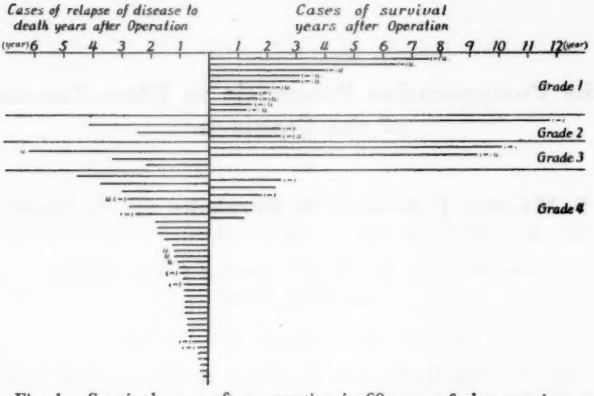
Cases of relapse of disease to death years after Operation
Cases of survival years after Operation
(year) 6 5 4 3 2 1
1 2 3 4 5 6 7 8 9 10 11 12(year)
Grade 1
Grade 2
Grade 3
Grade 4

Fig. 1. Survival years after operation in 60 cases of ulcer-carcinoma, as classified according to the depth of carcinomatous infiltration (grades 1 to 4). u, a case which was operated on an ulcer. (−), a case in which no lymph-nodal metastasis was found.

In 2 of 5 cases of grade 2, in which cancer tissue at the ulcer margin infiltrated the muscular layer but not the ulcer base, the patients died after 4 years 2 months and 1 year 8 months, respectively. These 2 cases of death were operated on for suspicion of ulcer-carcinoma, and lymph-nodal metastasis was confirmed microscopically. In the remaining 3 cases the patients are healthy at present, after postoperative 2 years, 2 years 3 months, and 11 years 9 months, respectively. The last case of 11 years 9 months survival was operated on for " gastric cancer," and no lymph-nodal metastasis was found.

Of 5 patients of grade 3, in which cancer tissue at the ulcer margin infiltrated the subserous layer, 2 are healthy at present, after postoperative 10 and 9 years, respectively; no lymph-nodal metastasis was observed. The remaining 3 patients died after postoperative 6, 3, and 2 years, respectively; lymph-nodal metastasis was observed in these 3 cases.

Of 40 cases of grade 4, in which cancer tissue infiltrated a great part of the ulcer base and groups of cancer cells were found beyond the gastric wall as well as in the normal omentum adjoining the callous omentum composing the base, 35 cases were operated on for " gastric cancer," and in 32 cases cancer cells were detected in resected lymph glands. Cases of death have amounted to as many as 34 in this group, the deaths occurring after more than 3 years postoperatively in 3 cases, after 2 to 3 years in 3 cases, and after about 1 year in 28 cases.

From what has been stated above it will be understood that in the group of cases (grades 1 to 3), in which infiltration of carcinoma originated

at the ulcer margin was confined to a small part of the base or to the subserous layer, late results of gastrectomy have been favorable, whereas the results have been unfavorable in the group of cases (grade 4), in which the infiltration extended to a great part of the ulcer base and beyond the serous membrane, namely, there are large differences in late results of gastrectomy between the groups of grade 1 to 3 and the group of grade 4.

Comparison of Late Results of Gastrectomy between Ulcer-Carcinoma and Gastric Cancer

Based upon the results mentioned above, our 60 cases have been divided into 2 groups, the group of grade 1 to 3 and the group of grade 4, and survival rates in successive years after operation in each group have been calculated, as shown in Fig. 2. It will be seen that the survival rates

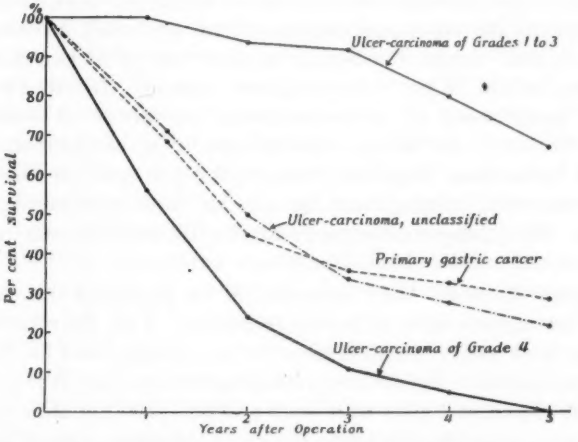

Fig. 2. Comparison of survival curves of ulcer-carcinoma cases with those of gastric cancer cases.

were 100, 93.8, 91.7, 80.0, and 66.7 per cent after postoperative 1, 2, 3, 4, and 5 years, respectively, in the former group of grades 1 to 3, while they were 55.0, 24.2, 11.1, 4.8, and 0 per cent after postoperative 1, 2, 3, 4, and 5 years, respectively, in the latter group of grade 4, thus showing much lower rates than in the former group.

When the cases of ulcer-carcinoma are not classified and considered as a whole, we obtain survival rates as follows: 70.0, 46.9, 35.9, 29.0, and 22.2 per cent after postoperative 1, 2, 3, 4, and 5 years, respectively. The survival curve drawn from these data is found to approximate the corresponding one obtained with 422 cases of resection of gastric cancer in

our clinic, as shown in Fig. 2, though a slight difference in the five year survival rate will be seen between the two curves (22.2 per cent for ulcer-carcinoma, and 28.9 per cent for gastric cancer).

Discussion

Finsterer[2-5] repeatedly stressed that the postoperative prognosis for ulcer-carcinoma is more unfavorable than for gastric cancer. According to him, even in cases of early ulcer-carcinoma where operation was performed for "ulcer" and malignant degeneration was detected first by microscopic examination, about one half of the patients died within 5 years with relapse of the malignancy, the 5-year survival rate being 51.4 per cent. In cases which were suspected or diagnosed as ulcer-carcinoma during operation, even if the operation was as radically as for primary cancer, the 5-year survival rate was as low as 9.3 per cent, making a striking contrast to the corresponding rate (29.6 per cent) in cases of gastric cancer. Ayabe[6] reported a 3-year survival rate of 33 per cent for ulcer-carcinoma, and of 34 per cent for gastric cancer. In our cases, the late results of gastrectomy in ulcer-carcinoma are similar to those in gastric cancer. However, the 5-year survival rate in ulcer-carcinoma (22.2 per cent) was lower than in gastric cancer (28.9 per cent) as Finsterer points out. At any rate, judging from the survival rates in successive years after operation, the postoperative prognosis for ulcer-carcinoma may be said to be nearly as unfavorable as for gastric cancer.

Recently Ranson[7] have reported, in his paper on the cancer of the stomach, favorable results of ulcer-carcinoma. This discrepancy seems to be due, at least partly, to differences in the criteria used for the diagnosis of ulcer-carcinoma. In our cases, the postoperative survival rates in ulcer-carcinoma of grades 1 to 3 were 91.7 and 66.7 per cent after postoperative 3 and 5 years, respectively, while those in ulcer-carcinoma of grade 4 were 11.1 and 0 per cent after postoperative 3 and 5 years, respectively. If the cases of grade 4, in which carcinomatous infiltration extended to a great part of the ulcer base, had been excluded from the cases of ulcer-carcinoma for deviating from the criteria of Hauser,[8] the survival rate would have become much higher than it was, thus pointing to a favorable prognosis. It is, however, generally accepted that ulcer-carcinoma does exist. Since it is a carcinoma, ulcer-carcinoma need not be restricted to the ulcer margin for long, and there may well exist an ulcer-carcinoma which has extended from the margin to its environs. Thus, from a viewpoint near that of Aschoff,[9] those cases of infiltration of the deeper part have been judged as cases of ulcer-carcinoma, and classed as of grade 4. If this viewpoint is adopted, our late results of operation, including those from grade 4 cases,

tell us that the prognosis for ulcer-carcinoma is as unfavorable as for gastric cancer.

Finsterer[2),5)] mentioned the following reasons why the prognosis for ulcer-carcinoma is more unfavorable than for gastric cancer: There is a strong tendency for ulcer-carcinoma to make a growth of infiltrating type; cases of ulcer-carcinoma may often be operated on with the clinical diagnosis of "ulcer," where the great omentum plays some part in relapse of malignancy; and lymph glands are liable to earlier involvement. This can be approved also from the results of our investigation. As a reason from the unfavourableness of ulcer-carcinoma prognosis, however, we should like to emphasize the frequent occurrence of cases of grade 4 (infiltration of the deeper part), namely, those cases which are liable to peritoneal carcinomatosis in spite of the small size of the cancer focus in the mucous membrane at the ulcer margin. This has been pointed out in our clinic these two years,[1),10-12)] and the conviction has been deepened with increase of cases. Briefly speaking, as the base of callous ulcer is made up of callous omentum, and the mucous membrane of the ulcer margin, where carcinoma originates, becomes closer to the omentum of the base; when there takes place rapid carcinomatous infiltration at the ulcer base, therefore, it is regarded as incipient peritoneal carcinomatosis. According to Kuru,[13)] frequent occurrence of highly malignant carcinoma simplex of scirrhous type, and a rapid deep carcinomatous infiltration of a poorly resistive established scar tissue containing few elastic fibers make the postoperative prognosis for advanced ulcer-carcinoma unfavorable.

From what has been stated above it goes without saying that cases of callous ulcer should be operated on before carcinoma develops, and that, operation for callous ulcer should be performed as radically as for carcinoma where resection of omentum tissue over a wide range in the environs of the base and thorough cleaning of swollen lymph glands are particularly necessary.

Summary

An investigation has been made on the late results of gastrectomy with 60 cases of ulcer-carcinoma where operation was performed more than one year ago, with the results to be summarized as follows: In cases of ulcer-carcinoma of grades 1 to 3 of deep infiltration, late results of operation are favorable, whereas the results are very unfavorable in cases of ulcer-carcinoma of grade 4.

References

1) Majima, Tohoku J. Exp. Med., this volume, p. 63.
2) Finsterer, Arch. klin. Chir., 1924, **131**, 71.

3) Finsterer, Wien. med. Wschr., 1927, **77**, 1342.
4) Finsterer, *ibid.*, 1938, **88**, 13.
5) Finsterer, Proc. Roy. Soc. Med., 1938–39, **32**, 183.
6) Ayabe, Igaku (Jap.), 1949, **6**, 268.
7) Ranson, Surg. Gyn. Obstr., 1953, **96**, 275.
8) Hauser, Handb. spez. path. Anat. u. Hist. (Henke u. Lubarsch), Berlin, 1926, **4**, 497–518.
9) Aschoff, Schweiz. med. Wschr., 1939, **20**, 261.
10) Abe, Majima & Kurakake, Nihon Geka Gakkai Zasshi (Jap.), 1952, **53**, 501.
11) Abe, Majima & Kurakake, Igaku (Jap.), 1952, **8**, 280.
12) Abe, Majima & Kurakake, Nippon Shokakibyogakkai Zasshi (Jap.), 1953, **50**, 9, 25.
13) Kuru, Geka (Jap.), 1953, **15**, 1.

The Tohoku Journal of Experimental Medicine, Vol. 62, No. 1, 1955

Chemistry and Biology of Lipids

XX. Interconversion *in vitro* of Blood Groups A and B*

By

Hajime Masamune and Ziroh Akama

(正 宗 一) (赤 間 二 郎)

From the Medico-chemical Institute, Tohoku University, Sendai

(Received for publication, April 19, 1954)

Specimens of the Group B lipoid of pig lung, prepared in particular wise, were found by chance to become Group A active as well with decrease of Group B activity on standing in contact with moisture. When its solutions of various pH values were stood at 10–40°C for 24 hours, the conversion was most marked at pH 7.0 and 40°C. On further standing of the solutions at the same temperatures, the both group activities diminished gradually in general, and on placing them in a refrigerator (0°C) on the other hand, the B potency recovered to the original strength with simultaneous disappearance of the A potency. Moreover the A and B potencies of a neutral solution stood at 40°C could be nullified separately by addition of normal α and β agglutinins respectively.

Hakomori[2] established in Group A lipoid of human liver that the carbohydrate moiety acting as the determinant group is linked with the aglycon of non-carbohydrate nature N-glycosidically. The same is analogized to hold in the present group lipoid possessing a resembling composition. Furthermore, since the A and B activities could be neutralized separately by α and β agglutinins respectively, formation of a non-specifically anti-agglutinative structure has been disproved in the present group conversion. Therefore, our discovery suggests that one of the broad surfaces (provisional right side) of the carbohydrate residue in the group substance is shielded by the aglycon, the other surface (wrong side) being left bare for combining with β agglutinin, and that the appearance of the Group A activity owes to the detachment of the right side of the carbohydrate from the aglycon by energy absorption, accompanied by anomerization of the N-glycoside and following loose attachment of the carbohydrate at the wrong side to the aglycon. If then, the later gradual decrease of the both group activities is regarded as due to scission of the N-glycosidic link

* Read before the 2nd General Meeting of the Japanese Union of Physiological Sciences, Tokyo, Nov. 10, 1952. A preliminary communication has been published in 1953[1].

(Cf. Tsuiki[3]), and the restoration of Group B activity, when the solution was chilled, is ascribed to the increase of the number of the molecules, whose determinant group is fixed in the original mode, by loss of energy, entailing shift of the equilibrium, either direct or indirect, between them and those whose determinant group is fastened at the wrong side toward themselves.

Experimental

Preparation of the Group B Lipoid

It was prepared by following Masamune and Hirata[4] with unavoidable modifications as follows.

Fresh lungs of 11 pigs weighing 2978 g. in all were washed with water to remove blood, freed from bronchi and bronchioles, and after grinding in a masticator, placed in 4.5 volumes of 91% alcohol containing 3.2% methanol at room temperature (10–20°C) for 1 week. The pulp was then squeezed and dried in a Faust apparatus. 519 g. was the dry material given. 400 g. of it were powdered in an iron mortar, boiled under reflux with 5 volumes of 96% undenatured alcohol for 3 hours and filtered warm. The mass on the filter was subjected again to similar extraction but with 3.5 volumes of the alcohol. The alcoholic solutions were united and stood in the cold for 24 hours, and the precipitate was centrifuged off. The clear, dark red supernatant was distilled in vacuo to syrupy consistency and kept overnight as covered under dry acetone. It was further washed with changes of the acetone until no more coloration of the acetone occurred, and placed in a vacuum desiccator ($CaCl_2$). 15 g. of a dark brown gelatinous substance were given. Originally it was intended to get a large amount of the group lipid for the structural study so that batches of this fraction were prepared by many runs, of which in some, the alcoholic solution and washing were condensed by distillation under ordinary pressure in the beginning and later under reduced pressure, and in some, extraction was effected with 96% alcohol denatured with methanol. The batches were separately preserved in vacuo over $CaCl_2$ for 1 week to 3 months. Thereafter, all of them excepting one were put together and preserved in a bottle tightly stoppered with a cork at room temperature (10–20°C) for about 4 months for certain circumstances. Unexpectedly the material (**Ia**) lost Group B potency (Nor Group A potency was recognized), becoming sticky due to absorption of moisture, contrary to the batch kept in vacuo (over $CaCl_2$) continuously (**Ib**), which also lost the B group potency when stood in air (15°C) for 24 hours (S. Table I). Many trials to restore its Group B potency failed, but we hesitated to throw it away and left it in a vacuum desiccator ($CaCl_2$). Nevertheless, when it

TABLE I

Group Potencies of the Mixture of the Alcoholic Tissue Extracts Stood in Contact with Some Moisture (**Ia**) and of the Extract Kept Avoiding Moisture (**Ib**)

The technique of the assay here was the same as is described in the section regarding the properties of a final fractionation product. **Ib** was assayed immediately (1) and after placing for 24 hrs. in air (2). − : no hemagglutination, ± : faint aggl., + : aggl.

Extract		Group of red cells	Dilution of the substances (1:)				
			5×10	10^2	2×10^2	4×10^2	8×10^2
Ia		A	+	+	+	+	+
		B	±	+	+	+	+
Ib	1	A	+	+	+	+	+
		B	−	−	−	+	+
	2	A	+	+	+	+	+
		B	+	+	+	+	+

was examined one day after about 4 months for caution's sake, it proved again Group B active to our surprise (Table II). Being thus encouraged,

TABLE II

Group Potencies of **Ia** after Standing in a Vacuum Desiccator for 4 Months and of **Ib** Kept in a Desiccator Continuously

Technique of the assay the same as remarked in Table I. Symbols − and + also signify the same as in Table I.

Extract	Group of red cells	Dilution of the substances (1:)				
		5×10	10^2	2×10^2	4×10^2	8×10^2
Ia	A	+	+	+	+	+
	B	−	−	−	+	+
Ib	A	+	+	+	+	+
	B	−	−	−	+	+

we resumed the fractionation of the lipid mixture, taking Group B specificity as an indication (Regarding the technique of blood group assay, see the section regarding the properties of a final product). Since many runs were carried out, a typical example (Run I) will be described in detail in the following. Namely, 5 g. of the substance were taken and extracted with four 50 cc. portions of redistilled ether. The red-brown solutions were distilled at 25°C to expel off the solvent as far as possible, and the remainder was treated with dry benzene in a similar manner. The benzene-

soluble part, recovered by distillation of the solution at 37°C, was dried after thorough washing with changes of acetone. The yield here was 2.9 g. This was subdivided by the aid of 200 cc. in total of dry ether. The ethereal solutions were distilled to about 5 cc., precipitated with 40 cc. of abs. alcohol and washed well with abs. alcohol and acetone in turn. The brownish powder given weighed 1.9 g. In the next, it was dissolved in 50 cc. of water (pH of the solution 5.0), added to with 0.1 *N* NaOH to pH 9.6 and shaken with an equal volume of redistilled benzene in a separatory funnel. The watery layer, that separated on standing, was further treated with two 50 cc. portions of benzene, and thereafter, acidified to pH 1.2 employing 1 *N* H_2SO_4 at first and 0.1 *N* H_2SO_4 afterward, whereby the solution became turbid, followed by five extractions with an equal volume of benzene (When a mixture did not separate into layers on standing, NaCl crystals were added). The benzene solutions were concentrated by vacuum distillation to a syrup and dehydrated and washed with acetone. 1 g. of a powder with yellowish hue was obtained. It was again taken up in water. The volume of water used this time was 25 cc. The solution which was of pH 4.2 was added to with sulfuric acid to pH 1.2 as above and the substance was driven into chloroform (Five 30 cc. portions of chloroform were employed) as into benzene. The chloroform solutions were condensed together by distilling at 37°C to 5 cc. or so and 10 volumes of acetone were added. The deposit was washed with acetone many times and dried in a vacuum desiccator ($CaCl_2$). 800 mg. of a yellowish powder were yielded. For quantitative analysis a portion was maintained long in the desiccator to a constant weight.

Properties of the Final Product in Run I

It showed one single boundary after electrophoresis at pH 7.8 (Fig. 1). It had solubilities in Table III. Molisch test resulted in positive, but biuret and Osaki-Turumi (the direct as well as the indirect) tests in negative. Analyses: N (micro Kjeldahl) 2.29%; P (modified Plimmer) 3.88%; ash (Pregl without use of H_2SO_4) 2.9%.

Group specificity. Two ranges of 0.2 cc. portions of 1% (neutral) and progressively diluted solutions of the substance in physiological saline were prepared, and in each range, 0.2 cc. of either normal anti-A or normal anti-B serum, preveously diluted to titre 16, was added to each of the portions. The mixtures were stood at 37°C for 2.5 hours with occasional agitation and at 0°C for 3 hours in succession. They were then added to with 2 drops of a 2% A or B erythrocyte suspension and stood in a thermostat at 37°C for 1 hour shaking at intervals of 15 minutes. In the latter stage, runs were carried out in parallel, replacing 0.4 cc. of the sera, same as above but diluted to titre 8 and progressively doubled, for

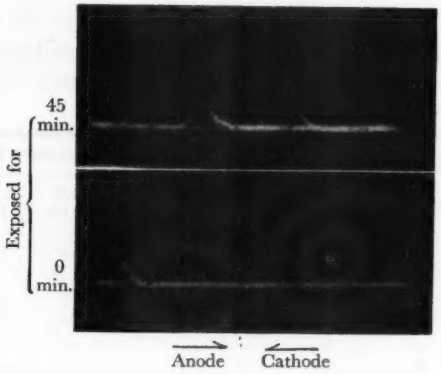

Fig. 1. Electrophoresis pattern of a 1% solution of the final product in Run I in phosphate buffer pH 7.8, I=0.20. Temperature 10°C; current 8 mA. Ascending limb to the left.

TABLE III

Solubilities of the Final Fractionation Product in Run I

−: insoluble, ±: sparingly soluble, ++: mostly soluble, ###: perfectly soluble.

Temperature	Solvent								
	Ether	Petrol ether	Methanol	Ethanol	Chloroform	Benzene	Water		
							acidified	plain	alkalized
Cold*	−	−	−	−	###	###	++	++	###
Warm†	−	−	±	±	###	###	++	++	###

* Not heated. † Heated for a short while on an electric heater.

the mixtures of the substance and sera above, and judgement of the results was made by determining by means of a magnifier the dilutions of the substance where it inhibited hemagglutination, when the agglutination took place in the third test tubes but not in the fourth in the respective reference runs. Table IV shows the results not only with the product in Run I but also with those in other runs.

Experiments Concerning Group Conversion

Group potency was assayed as described in the above section, excepting in the experiment in 4) below.

TABLE IV

Group Potencies of the Final Fractionation Products

Technique of the assay the same as remarked in Table I. Symbols −, ± and + signify also the same as in Table I.

Run wherein the product was given	Group of red cells	Dilution of the substances (1:)							
		10^2	2×10^2	4×10^2	8×10^2	1.6×10^3	3.2×10^3	6.4×10^3	1.28×10^4
I	A	+	+	+	+	+	+	+	+
	B	−	−	−	−	−	−	+	+
II	A	−	±	+	+	+	+	+	+
	B	−	−	−	−	−	±	+	+
III	A	+	+	+	+	+	+	+	+
	B	−	−	−	−	−	±	+	+
IV	A	±	+	+	+	+	+	+	+
	B	−	−	−	−	−	−	+	+
V	A	+	+	+	+	+	+	+	+
	B	−	−	−	−	−	+	+	+

Since the Group B activity of the alcoholic tissue extract vanished on long standing without strict avoidance of air, a small part of the preparation above was exposed to air for about 2 days at 15°C to see if we encounter a similar happening as in the material **Ib**. Strange to say, Group A activity appeared with decrease of the Group B activity (Table V), so that the following experiments were undertaken to investigate the mechanism of the probable group interconversion.

TABLE V

Group Activities of the Final Product in Run I Examined after Standing it in Air at 15°C for 44 Hours (1) and Immediately (2)

Technique of the assay the same as remarked in Table I. Symbols −, ± and + signify also the same as in Table I.

Examination	Group of red cells	Dilution of the substance (1:)							
		10^2	2×10^2	4×10^2	8×10^2	1.6×10^3	3.2×10^3	6.4×10^3	1.28×10^4
1	A	−	−	±	+	+	+	+	+
	B	−	−	−	±	±	+	+	+
2	A	+	+	+	+	+	+	+	+
	B	−	−	−	−	−	−	+	+

The data below are picked out from the experiments on the product in Run I of preparation. The results with the corresponding products after other runs were resembling.

1) *Effect of pH of the solution on the group conversion.* 0.5 cc. portions of a 1% solution in physiological saline were adjusted to various pH values with 1/100–1/10 *N* NaOH solutions as follows:

pH values to which the portions were adjusted	Drops added of NaOH at			pH of the portions after 24 hrs. at 10°C
	0.01 *N*	0.02 *N*	0.1 *N*	
2.0	0	0	0	1.6
3.0	3	0	0	2.8
4.0	4	1	0	4.0
5.0	1	0	2	4.6
6.0	1	1	2	6.0
7.0	0	1	3	6.8
8.0	3	1	3	7.4
9.0	0	1	4	8.4

TABLE VI

Effect of pH on Group Conversion

Solutions of various pH values of the product in Run I were stood for 24 hrs. at 10°C. Technique of assaying group potency was the same as remarked in Table I. Symbols −, ± and + signify also the same as in Table I. (∓) signifies scarce agglutination. The acidic solutions were cloudy.

Solution		Group assay								
		Group of red cells	Dilution of the substance (1:)							
No.	pH		10^2	2×10^2	4×10^2	8×10^2	1.6×10^3	3.2×10^3	6.4×10^3	1.28×10^4
1	2.0	A	+	+	+	+	+	+	+	+
		B	−	−	−	−	−	−	−	+
2	3.0	A	+	+	+	+	+	+	+	+
		B	−	−	−	−	−	−	−	+
3	4.0	A	+	+	+	+	+	+	+	+
		B	−	−	−	−	−	−	−	+
4	5.0	A	+	+	+	+	+	+	+	+
		B	−	−	−	−	−	−	±	+
5	6.0	A	−	−	−	(∓)	±	+	+	+
		B	−	−	−	−	−	−	+	+
6	7.0	A	−	−	−	−	±	+	+	+
		B	−	−	−	−	−	(∓)	+	+
7	8.0	A	−	−	±	±	+	+	+	+
		B	−	−	−	−	±	+	+	+
8	9.0	A	−	−	±	±	+	+	+	+
		B	−	−	−	−	+	+	+	+
Control*		A	+	+	+	+	+	+	+	+
		B	−	−	−	−	−	−	−	+

* The substance was assayed immediately without prior standing of its neutral solution.

And they were stood at 10°C for 24 hours as stoppered with a cork. They were followingly neutralized with NaOH or HCl, and after filling up to 0.75 cc. with water, supplemented with 1.8% NaCl so as to obtain 1 cc. of solutions in physiological saline. As shown in Table VI, most significant change of group activity occurred at pH 7.0. At pH values of 2.0 to 5.0 no alteration was witnessed.

2) *Effect of time of standing the solution* (Table VII). 1% solutions of pH 3.0, 7.0 and 9.0 were stood at the same temperature as above for 72 hours.

TABLE VII

Effect of Time on Group Conversion

Solutions of the final product in Run I were stood for various durations of time at 10°C. Technique of assaying group potency was the same as remarked in Table I. Symbols −, ± and + signify also the same as in Table I. The solution at pH 3.0 was cloudy.

Solution			Group assay								
				Dilution of the substance (1:)							
No.	pH	Time of standing in hrs.	Group of red cells	10^2	2×10^2	4×10^2	8×10^2	1.6×10^3	3.2×10^3	6.4×10^3	1.28×10^4
1	3.0	24	A	+	+	+	+	+	+	+	+
			B	−	−	−	−	−	+	+	+
		48	A	−	−	±	+	+	+	+	+
			B	−	−	−	−	±	+	+	+
		72	A	±	±	+	+	+	+	+	+
			B	−	−	−	−	±	+	+	+
2	7.0	24	A	−	−	−	−	+	+	+	+
			B	−	−	−	−	−	+	+	+
		48	A	−	−	−	±	+	+	+	+
			B	−	−	−	−	±	+	+	+
		72	A	−	±	+	+	+	+	+	+
			B	−	−	−	±	+	+	+	+
3	9.0	24	A	−	−	+	+	+	+	+	+
			B	−	−	−	±	+	+	+	+
		48	A	−	−	−	±	+	+	+	+
			B	−	−	±	+	+	+	+	+
		72	A	−	−	−	±	+	+	+	+
			B	−	±	±	+	+	+	+	+

i) The solution at pH 7.0 was most Group A potent among others after 1 day. In this solution both the A and B activities disappeared gradually since then. ii) At pH 9.0, A activity arose slowly and decrease of the B activity was quicker than at other pH values. iii) At pH 3.0, Group A activity developed only after 2 days and was no more detected

after 3 days. Group B activity lowered in similar rate as at pH 7.0.

In short, appearance of Group A activity is most marked and quick at pH 7.0, and on the other hand, not only Group A activity once generated but also Group B activity diminishes with time at any of the pH values.

3) *Effect of temperature.* At first, portions of a 1% solution at pH 7.0 were stood for 1 day at various temperatures 0°, 20° and 40°C. At 0°C almost no change was observed, but it occurred distinctly at 20°C and more rapidly at 40°C (Table VIIIA). Next, another 1% solution at pH 7.0, stood at 40°C for 1 day, was placed in a refrigerator and its group potencies were estimated from time to time. The Group A activity, which had arisen by exposure to 40°C, became less and less, Group B potency increasing simultaneously, and the original Group B specificity was restored after 4 days (See Table VIIIB).

4) *Nullification of the Group A and B activities with α and β agglutinins respectively* (The writers are indebted to Dr. T. Miki in the Forensic Medical

TABLE VIII

Effect of Temperature on Group Conversion

Technique of assaying group potency was the same as remarked in Table I. Symbols −, (∓), ± and + signify the same as in Table VI.

Solution				Group assay								
No. of portions	pH	Temperature °C	Time of standing in hrs.	Group of red cells	Dilution of the substance (1:)							
					10^2	2×10^2	4×10^2	8×10^2	1.6×10^3	3.2×10^3	6.4×10^3	1.28×10^4
A. When portions of a solution at pH 7.0 of the product in Run I were stood for 24 hrs. at various temperatures												
1	7.0	0	24	A	±	±	+	+	+	+	+	+
				B	−	−	−	−	−	±	+	+
2		20		A	−	−	−	±	+	+	+	+
				B	−	−	−	−	−	+	+	+
3		40		A	−	−	−	−	+	+	+	+
				B	−	−	−	±	+	+	+	+
B. When a solution at pH 7.0 of the same substance, stood at 40°C for 24 hrs., was placed in a refrigerator at 0°C												
	7.0	40	24	A	−	−	−	−	+	+	+	+
				B	−	−	−	−	+	+	+	+
		0	24	A	−	−	−	±	+	+	+	+
				B	−	−	−	−	−	+	+	+
			48	A	−	−	(∓)	+	+	+	+	+
				B	−	−	−	−	−	±	+	+
			96	A	−	(∓)	±	+	+	+	+	+
				B	−	−	−	−	−	−	+	+

Institute) (Table IX).

TABLE IX

Nullification of the Group A and B Activities of the Product in Run I after Standing its Solution of pH 7.0 at 40°C for 24 Hours

Regarding the technique of group assay, see the text. Symbols −, ± and + signify the same as in Table I.

No. of experiments	Absorption		Group assay							
	Serum for nullifying either A or B activity (a)	Quantity of the antibody in (a)	Serum	Group of red cells	Dilution of the substance (1:)					
					10^2	2×10^2	4×10^2	8×10^2	1.6×10^3	3.2×10^3
i			A	A	−	−	−	±	+	+
			B	B	−	−	−	−	−	+
ii			O	A	−	−	±	+	+	+
				B	−	−	−	−	−	+
iii	O serum absorbed w. B erythrocytes	1 equiv. to A activity	A	A	+	+	+	+	+	+
			B	B	−	−	−	−	+	+
iv	"	3 equiv. to A activity	A	A	+	+	+	+	+	+
			B	B	−	−	−	+	+	+
v	O serum absorbed w. A erythrocytes	1 equiv. to B activity	A	A	−	−	−	+	+	+
			B	B	+	+	+	+	+	+
vi	"	3 equiv. to B activity	A	A	−	−	±	+	+	+
			B	B	+	+	+	+	+	+

i) Two 0.1 cc. portions of a neutral 1% solution of the substance which had been stood at 40°C for 1 day were progressively diluted and added to with 0.1 cc. of normal anti-A or anti-B serum, priorily diluted to titre 8, followed by standing at 37°C, at room temperature and at 0°C for 1 hour each in succession with occasional shaking. Thereupon, hemagglutination inhibition was examined after placing 1 drop of the mixtures and 1 drop of a 1% A or B erythrocyte suspension together in a hollow objectglass for 30–45 minutes at room temperature.

ii) An O serum possessing α and β agglutinins of equal titres was diluted to titre 8 of the antibodies and used in stead of the diluted B and A sera in i).

The results in i) and ii) which were similar to each other indicate the Group A and B potencies of the lipid treated as described.

iii) 0.5 cc. of the lipid solution above was mixed with 0.5 cc. of the O serum, which had been freed from β agglutinin with B erythrocytes and so diluted as to contain a quantity of α equivalent to the A potency of the lipid, and stood at 37°C, room temperature and 0°C in turn for 1 hour each, followed by determination of the group potencies as in i). The A

activity was no more recognized at the dilution 1:100 of the substance, but the B activity also lowered more or less.

iv) A similar experiment as in iii) was processed with the absorbed O serum which, however, had been less diluted that it might contain *a* agglutinin three times as concentrated. The A activity was nullified as in iii), but the decrease of B activity was more noticeable.

v) and vi) Experiments were carried out similar to those in iii) and iv), employing the O serum absorbed with A erythrocytes in place of B erythrocytes. As in iii) and iv), the agglutinin used neutralized the corresponding group activity with some simultaneous decrease of the A potency.

The results that the Group B potency was weakened in iii) and iv) and the Group A potency in v) and vi) may be explained by partial transformation of the structures exhibiting A and B activities into those exhibiting the B and A activities respectively to attain equilibrium directly or through an intermediate structure or structures.

Through the Grant Committee for Scientific Researches the Ministry of Education gave a grant in aid to us. H. Masamune.

References

1) Masamune, Tohoku J. Exp. Med., 1953, **57**, 310.
2) Hakomori, *ibid.*, 1954, **60**, 331.
3) Tsuiki, *ibid.*, 1955, **61**, 267 and 365.
4) Masamune & Hirata, *ibid.*,-1952, **55**, 233.

The Tohoku Journal of Experimental Medicine, Vol. 62, No. 1, 1955

Studies on Tuberculous Tracheobronchitis

I. Experimentally Established Tuberculous Bronchial Ulcer in Dog

By

Katsumi Kozu

(神津 克己)

From the National Kiyose Sanatorium, Tokyo

(Received for publication, April 19, 1954)

Reports on the pathogenesis of tuberculous lesions in the trachea and major bronchi have been made by many workers[1),2),3),4),5)]. Considering their theories synthetically, the mode of development of tuberculous tracheobronchitis will be classified, as stated by Jenks[6)], into four: (i) implantation or direct contact, (ii) continuity, (iii) contiguity, and (iv) haematogenous dissemination. Among these, the mode of implantation, in which tuberculous tracheobronchitis develops by means of the direct invasion of tubercle ßacilli attached on tracheobronchial mucosa through mucous epithelium or by way of ducts of the mucous glands, was considered most important by Auerbach[7),8)], Bugher, Littig and Culp[2)], Huang[3)], Shimamura and Yoshida[9)], Ninomiya[10)] and others.

These theories on the mechanism of the development of tuberculous tracheobronchitis have been based on the bronchoscopic and pathologic findings in human body. From the standpoint of the mode of implantation, the author has tried to establish experimentally the bronchial ulcer in animals by infecting its tracheobronchial mucosa directly with tubercle bacilli; that is, tubercle bacilli were attached on the mucous membrane or injected into the mucous tissue of the bronchus of dogs, and after the change in the mucous membrane was observed bronchoscopically, the lesion was examined histologically to clarify the development of the tuberculous bronchial ulcer.

Experimental

Material and Methods

48 dogs aged 1 to 2 years and reacting negatively to Römer's testing were employed. They weighed 14 to 16 kg. By use of a bronchoscope 9 mm. in inside-diameter and 50 cm. in length, the inside of the inner wall of their left main bronchi was infected by various methods with virulent tubercle bacilli of human type (Kiyose H_1 strain), and the change

in bronchial mucosa was examined bronchoscopically once or twice every week. For some groups, percutaneous injection was done into the lung to observe bronchoscopically the changes of the bronchial trunk of regional lobes.

Bronchoscopy was done after anaesthesia by femoral-venous injection with 3 to 4 cc. of Evipal (cyclopan-natrium). Lastly, all of these dogs were killed with bleeding, and examined from macroscopic inspection at necropsy and microscopic sections.

According to the method of infection, four kinds of experiments were done, each consisting of 1 to 3 groups, totalling 8. The number of dogs employed in each group was 5 to 7.

Experiment I. Painting of tubercle bacilli on the bronchial mucosa

Group A: Simple painting of bacilli

A long cotton wool holder was soaked in 50 mg./cc. to 100 mg./cc. emulsion of tubercle bacilli using 50% glycerine solution, and through the bronchoscope, this solution was painted on the mucosa of the left inner wall of the main bronchus. Since no change was observed bronchoscopically after a painting, the same manner was repeated twice a week for a period of 5 weeks.

Group B: Painting of bacilli after applying arsenious-acid paste

Arsenious-acid paste was previously applied on the bronchial mucosa twice at 3 day interval to produce inflammation on the membrane, and then the same procedure as described in Group A was conducted.

Experiment II. Painting of tubercle bacilli in combination with curetting

By the use of a long metal stick the end of which was three-forked with sharpened hook, the inside of inner wall of main bronchus was curetted in such a manner as it bleeds slightly. On this site, the same bacilli as employed in Experiment I was painted. Since the reaction of the painted site subsided 1 to 2 weeks later, this procedure was repeated once a week for 5 weeks.

Experiment III. Submucosal injection of tubercle bacilli

Group A: Single injection

30 mg. (1.3 cc. of 20 mg./cc.) of tubercle bacilli of human type was injected submucously into the several sites of the same part as described in Experiments I and II, and 5 weeks' observation was performed.

Group B: Repeated injections

The same procedure as in Group A was repeated once a week for 5 weeks.

Group C: Single injection after abdominal injection of bacilli

40 mg. (2 cc. of 20 mg./cc.) of the above-mentioned bacilli was injected into the abdomen of dogs, and 1 to 4 weeks later, a submucous injection into

the main bronchus was done as in Group A.

Experiment IV. Pulmonary injection

The orifice of the left upper lobe bronchus was given a slight scratch with the curetting stick as used in Experiment II. Then, 30 mg. (1.5 cc. of 20 mg./cc.) of bacilli was injected into the left upper lobe through he. third intercostal space on the left anterior axillary line, and the change at the orifice of the bronchus was observed.

Group A: Short-term observation

The above procedure was repeated once a week for 5 weeks.

Group B: Relatively long-term observation

The amount and the number of times of injection were reduced in order to make the dogs live longer and also to continue the observation for a longer time than in Group A. 10 mg. of bacilli was injected into the lung of 5 animals twice at an interval of a week. Three out of those 5 animals were given an additional injection one month later.

Roentgenographic examination was made more frequently in this group than in others for the purpose of examining the relationship between the change in bronchial mucosa and pulmonary lesions together with cavity formation.

Results

Table I shows the summary results of bronchoscopic and pathologic findings on the inner wall of the main bronchus and of the orifice of the left upper lobe bronchus (in Experiment 4), and also of the examination for the presence of acid-fast bacilli in microscopic sections.

Experiment I. Painting of bacilli

In Group A, only a very slight hyperemia, edema or white thin secretion was observed through bronchoscopy on the mucosa at the painted site, and even after 5 weeks any marked change was not found. In Group B, the inflammation caused by arsenious-acid paste has almost subsided after 2 to 3 weeks. But, when tubercle bacilli were repeatedly painted before the inflammation became indistinct, the hyperemia and swelling of the mucosa were found very marked forming a granular stenosis.

Macroscopic findings at necropsy of some of the animals in Group A showed a slight rough change on the mucosa, while others showed no abnormal findings. In Group B, the lumen of the bronchus was very narrow (Fig. 1), and the surface of the mucosa was folded presenting small superficial ulcers at places.

Histological findings in Group A revealed a slight cell infiltration in the subepithelial and submucous tissues, and as a specific reaction, tubercles were discovered in some cases, while none in others. These tubercles

TABLE I

Bronchoscopic and Pathologic Findings in the Main Bronchus and Acid-fast Bacilli in Sections

Experimental groups		Bronchoscopic types	Pathologic findings			
			Macroscopic findings	Microscopic findings		
				Tubercle	Ulceration	Acid-fast bacilli
I	A	Slight inflammatory	Roughness (±)	(±)	(−)	(−)
	B	Fibrostenotic	Stenosis and ulceration	(+)	(++)	(+)
II		Ulcerative	Roughness and ulceration	(++)	(++)	(++)
III	A	Submucosal or ulcerative	Roughness, ulceration (±)	(++)	(±)	(+)
	B	Ulcerostenotic	Stenosis and ulceration	(+++)	(+++)	(+++)
	C	Submucosal or ulcerative	Roughness, ulceration (+)	(++)	(+)	(+)
IV	A	Slight inflammatory	Roughness	(−)	(−)	(−)
	B	Slight inflammatory	Roughness	(−)	(−)	(−)

Notes: ± represents pathologic findings negative or slightly positive.

Fig. 1. Case painted with tubercle bacilli after applying arsenious-acid paste (Exp. I, Group B). Photograph showing cicatrical stenosis of main bronchus in the dog.

were composed of epitheloid cells, the number of which was very little.

In Group B, a strong infiltration was observed all over the mucosa and submucous tissue; the formation of new blood vessels and hyperemia were evident, and the epithelium was very thin, sometimes broken down at the site of severe lesions. Here and there, ulcers were observed extending to the muscular layer, or tubercles were found in which a small number of round cells were mixed with epitheloid cells. A small amount of acid-fast bacilli was detected in the sections. These changes were especially; obvious on the surface of mucosa, and adventitia was free from the change.

Experiment II. Painting of tubercle bacilli in combination with curetting

Bronchoscopic findings 5 weeks after the painting of bacilli in combination with curetting revealed reddish ediematous swellngs upheaved irregularly on the mucosa of the inside of inner wall of the bronchus. This swelling easily bled by touching, and sometimes it was found forming a granulomatous ulcer. Any strong stenosis was not observed.

Macroscopic findings at autopsy showed an uneven rising, islandlike or linear (Fig. 2), and there were from some defects of the mucous epithelium as small as a spot to an ulcer as large as 15×30 mm.

Fig. 2. Case painted with tubercle bacilli in combination with curetting (Exp. II). Photograph showing the roughness of mucous membrane of main bronchus in the dog.

Histologically, the cell infiltration was found marked, partly softened and broken down, or forming an ulcer extending to the muscular layer. Epithelium was almost lost, or there was a thin loose epithelium broken down at places. Hyperplasia of the connective tissue was observed par-

tially. A considerable number of tubercles existed there, and in the sections of which acid-fast bacilli were proved moderately.

Experiment III. Local infection of bacilli

In Group A, hyperemia and uneven swelling were found bronchoscopically on the mucosa two to three weeks after the submucosal injection with tubercle bacilli. Then, these hyperemia and swelling reduced, and the degree of unevenness lessened, but its number increased, and in some cases, a granulomatous ulceration ready to bleed was formed four to five weeks later.

Autopsy disclosed that the local mucosa was rough presenting small risings like islands with or without several superficial ulcerations 2×5 mm. diameter round or spindle-like.

Histologically, the cell infiltration of mucous and submucous tissues was strong; these tissues were loose at the sites where the surface of mucosa was rising macroscopically, and new formation of blood vessels was marked there. On the submucous tissues, a considerable number of epitheloid cell tubercles were observed. Epithelial cells were partly fallen, and in some places mucous tissues turned into ulcer, the degree of which was not strong. Acid-fast bacilli were detected in the tissues.

In Group B, through bronchoscopical examination, hyperemia and swelling were observed covering the entire surface of the bronchus in the area which was repeatedly injected with tubercle bacilli. The injected site was remarkably rough, and was covered with ash-coloured pseudomembrane forming an excavated ulcer. The bronchial lumen presented inflammatory or granulomatous stenosis.

Necropsy revealed the stenosis of the bronchial wall (Fig. 3) caused by the thickness of the wall and the swelling of the bifurcation lymph nodes, and also the ulceration at the site of injection. The size of these ulcers was various, from the small superficial one to the large deep one, e.g., 10×15 mm. in width (Fig. 4).

Histologically, exudative cell infiltration was marked reaching to the submucous layer, while productive infiltration or caseous necrosis also existed in some parts. Some of the lesions were obviously hyperemic with bleeding at places. Part of the caseous necrosis was broken down into the bronchus forming ulceration. In the cases in which ulceration was very strong, the cartilage was broken down, exposing itself at the base of the ulcer (Fig. 5). There were many tubercles containing plenty of acid-fast bacilli. None of the giant cell of Langhans type was detected also in this group which produced the most severe lesion among the groups under the present study.

As to Group C in which abdominal injection was previously made with

Fig. 3

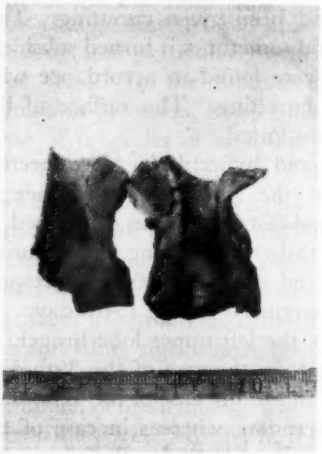

Fig. 4

Fig. 3. Case of local injection of tubercle bacilli (Exp. III, Group B). Remarkable stenosis due to the thickening of bronchial wall and the swelling of lymph nodes.

Fig. 4. Case of local injection of tubercle bacilli (Exp. III, Group B). Photograph showing the large deep ulcer in main bronchus in the dog.

Fig. 5. Microphotograph showing the ulcer of main bronchus of the case of local injection of tubercle bacilli (Exp. III, Group B). Cartilage is broken down at the base of the ulcer.

tubercle bacilli and one to four weeks later submucous injection was one into the inner wall of the main bronchus, similar results with those of Group A of this experiment were obtained showing no significant difference between them.

Experiment IV. Pulmonary infection of bacilli

In Group A, namely, short-term obseravtion group, hyperemia and

edema appeared two to three weeks after the first injection at the orifice of the left upper lobe bronchus which had been given curetting. This change sometimes continued for 5 weeks, and sometimes it turned subsided.

Marcroscopical findings at necropsy were found in accordance with those of the site which was subjected to curetting. The surface of the mucosa was granular, and no ulceration was found.

Histologically, slight cell infiltration and hyperplasia of connective tissues were recognized corresponding to the rising of the surface of mucosa, but none of tubercles, ulcers or acid-fast bacilli was discovered.

In Group B, observation was made rather for a long time, during which 3 animals died, on the 39th day, 42nd day and 100th day respectively. The remaining two animals were sacrificed on the 154th day.

Bronchoscopic findings at the orifice of the left upper lobe bronchi of these animals during their life showed that, in the case of the 39th-day-death the mucosa of the orifice was rough, and in the case of the 42nd-day-death the lumen was filled with foamy secretion, whereas in case of the 100th-day-death the mucosa was almost normal. As for the remaining two animals killed on the 154th day, hyperemia and edema were observed in one, while in the other, yellowish-white tenacious secretion was draining out of the orifice.

On all the roentgenograms, tuberculous pneumonia was found in the left upper lobe, and cavity was proved in two animals died on the 42nd day and the 100th day respectively (Fig. 6). Necropsy thereafter revealed a cavity formatiou also in one of those animals killed on the 154th day.

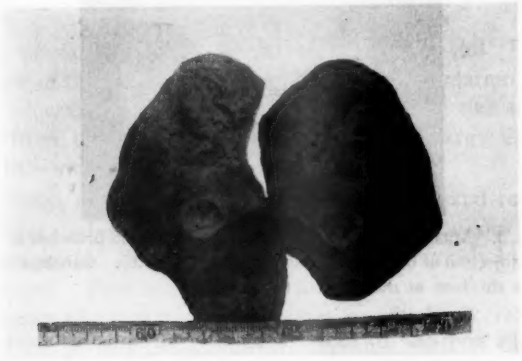

Fig. 6. Case of pulmonary injection of tubercle bacilli (Exp. IV, Group B). Photograph showing cavities in the left upper lobe in the dog.

At necropsy, the mucosa of the orifice of the left upper lobe was rough, but ulceration was not found. Histologically, epithelial cells of the mucosa

were found pseudonecrotic or necrotic, which were partly broken down. This change was marked chiefly with the animals that had cavitation in the lung, and particularly with those that showed draining secretion during their lives. In the submucous tissues, there were found a slight new formation of vessels, hyperemia, and hyperplasia of connective tissues, but none of tubercles or acid-fast bacilli existed there.

Toward the distal and of the drainage bronchus, the degree of necrosis and broken-down of the epithelial cells was gradually intensified, and the lumen of small bronchus was filled with larger necrotic mass and secretion. Change in mucous and submucous tissues was very slight without any reaction peculiar to tuberculosis. In the peripheral bronchioles without cartilage, the tuberculous infiltration of the neighboring pulmonary tissues was spreading right under the mucous epithelium, at places destroying the epithelium, and the cells composing pulmonary lesion were flowing out into the lumen of bronchioles. But in the case of small bronchi having cartilage, even if they were existing in the similar lesion as above, such changes were found stopping just under the bronchial adventitia giving no remarkable effect on the bronchial wall. Only the mucous epithelium was in strong necrosis, and this change clearly confirmed the fact that it occurred intracanalicularly out of the necrotic mass or secretion which was carried from the peripheral bronchioles.

The number of dogs died during the course of the present experiments was 7 in all, one each in Group B of Experiment I and in Experiment II, and Groups A and B of Experiment III, and three in Group B of Experiment IV. The cause of death is considered to be tuberculous infection in Experiment IV, and in others it is considered to be the Evipal anaesthesia given on the weak body after infection. They died following anaesthesia.

In both sides of their lungs, exudative lesions existed in various sizes from acinous-nodular to segmental sizes. These lesions were found, especially in the lungs of the main bronchus in which bacilli were injected. But contrary was found in some cases. Miliary dissemination was found in a case of Group B, and in two cases of Group C of Experiment III. Particularly marked findings were that lesions occurred frequently in the apex of the lower lobe.

Cavity formation was observed at the apex of the left lower lobe in one case of Group A of Experiment III, besides 3 cases of Experiment IV. In Experiment IV, cavity was found in the left upper lobe where bacilli had been injected, and where tuberculous pneumonic lesions existed in segmental to lobar sizes.

Referring to the swelling of hilar lymph nodes, it was most distinguished with bifurcation lymph nodes, next with epibronchial lymph nodes and

then with paratracheal lymph nodes. In the case of pulmonary injection group, however, the swelling of the bifurcation lymph nodes was very slight. Compression of the bronchus due to the swelling of lymph nodes was marked in Experiment III (particularly in Group B), but it was slight in other experiments.

Discussion

When the normal bronchial mucosa of dogs was painted with virulent tubercle bacilli of human type twice a week for a period of 5 weeks, no ulceration was found except very slight mucous lesions. However, in case infection was made on the inflammatory mucous membrane previously caused by arsenious-acid paste or on the scratched membrane, ulceration was found obviously. These results indicate that in the normal mucous membrane, ulceration is difficult to be made by simple painting of bacilli, whereas under such conditions as bacilli will easily invade into the mucosa through the inflammation or scratch, the area will be involved easily. In other words, for the development of tuberculous tracheobronchitis, the occurrence of locus minoris resistentiae will be considered important.

Next, when bacilli were injected into the submucous tissue, ulceration appeared always in the repeated injection group, whereas the local lesion tended to heal in the single injection group, the lesion becoming more severe by the additional injections. From this result, repeated invasions of tubercle bacilli will be considered as an another important factor for the development of tuberculous tracheobronchitis. It may have something to do with the local allergy. In order to examine its relationship with the general allergy, tubercle bacilli were injected into the abdomen and then additional injection was given submucously into the bronchus, with the results that any marked local reaction was not observed as compared with the control in which such a preliminary treatment was not done.

In order to establish the condition similar to tuberculous tracheobronchitis following pulmonary tuberculosis in man, bacilli were injected into the lung of the animals after the mucosa of the orifice of the lobe bronchus was given slight scratches so as the bacilli running out of the lung may easily adhere on the bronchial mucosa. The result was that, although any ulceration was not found, yet epithelial cells of the mucosa were discovered being involved and broken down successively and intracanalicularly starting from the bronchioles regional to the cavities or exudative lesions toward the larger bronchi.

The bronchus with cartilage was influenced by the surrounding tuberculous parenchymal lesions in the different manner from the bronchiolus

without cartilage, even if both of them were in the similar pulmonary lesions, that is, bronchiolus without cartilage was readily involved by the surrounding parenchymal lesions while the bronchus having cartilage was protected comparatively well.

As for the relation between the bronchial stenosis and the lymph node, the swelling of lymph node was very slight, peribronchial tissue was separated from lymph nodes, and histologically also adventitia was free in the group of the painting of tubercle bacilli after applying arsenious-acid paste which showed marked stenosis. So the stenosis of this case will be attributed to the cicatrical contraction caused by the strong hyperplasia of connective tissues of the bronchial wall.

On the other hand, in the repeated submucous injection group, the stenosis will be ascribed chiefly to the thickening of bronchial wall due to cell infiltration and compression by the swelling of surrounding hilar lymph nodes especially the bifurcation lymph nodes. In this case, lymph nodes were closely attached to the bronchi and adventitia was filled with cell infiltration. Here the secondary progress of bronchial tuberculosis through the lymphatics, as mentioned by Reichle and Frost, Schwartz[11] *et al.*, should be taken into consideration, but it was not clarified in this study.

Summary

From the view point of implanatation theory on the tuberculous tracheobronchitis, tuberculous bronchial ulcer was experimentally established by attaching tubercle bacilli of human type on the main bronchus of dogs by various methods.

1. In the normal bronchial mucosa, ulceration was not caused by repeated painting of tubercle bacilli on the site twice a week for a period of 5 weeks.

2. Ulceration was produced when bacilli painting was done on the inflammatory or the scratching site previously made on the mucosa.

3. Ulceration was caused always by repeated submucous injections of bacilli but not by a single injection.

4. From these results, locus minolis resistentiae and repeated invasions of bacilli are considered to be favourable factors for the development of tracheobronchial tuberculosis.

5. In case submucous injection with bacilli was tried one to four weeks after an abdominal injection, no marked difference was observed as compared with the case of similar injection without any preliminary procedure.

6. In the group of long period observation after pulmonary injection, mucous epithelial cells of the orifice of the lobe bronchus were found

necrotic or partly desquamating due to the attaching of secretion that brought tubercle bacilli from the pulmonary lesions especially from cavities. Peripheral bronchiolus-wall without cartilage was easily influenced by the surrounding pulmonary lesions, whereas the bronchus with cartilage was hardly affected.

7. When bacilli were painted after applying arsenious-acid paste, there was a marked bronchial stenosis which was mostly cicatricial, while in the case of repeated injections of bacilli, the stenosis was also marked owing to the thickness of mucous membrane and to the swelling of hilar lymph nodes.

References

1) Reichle, & Frost, Am. J. Path., 1934, **10**, 651.
2) Bugher, Littig, & Culp, Am. J. Med. Sc., 1937, **193**, 515.
3) Huang, Am. Rev. Tuberc., 1945, **47**, 500.
4) Silverman, Dis. Chest, 1945, **11**, 3.
5) Jones, & Alley, Am. Rev. Tuberc., 1951, **63**, 381.
6) Jenks, *ibid.*, 1940, **41**, 692.
7) Auerbach, Quart. Bull. Sea View Hosp., 1938, **3**, 134.
8) Auerbach, Am. Rev. Tuberc., 1949, **60**, 604.
9) Shimamura, & Yoshida, Kekkaku (Jap.), 1949, **24**, 433.
10) Ninomiya, Kekkaku-Sensho (Jap.), No. 4, 1952.
11) Schwartz, Forts. Tuberkuloseforsch., 1952, **5**, 255.